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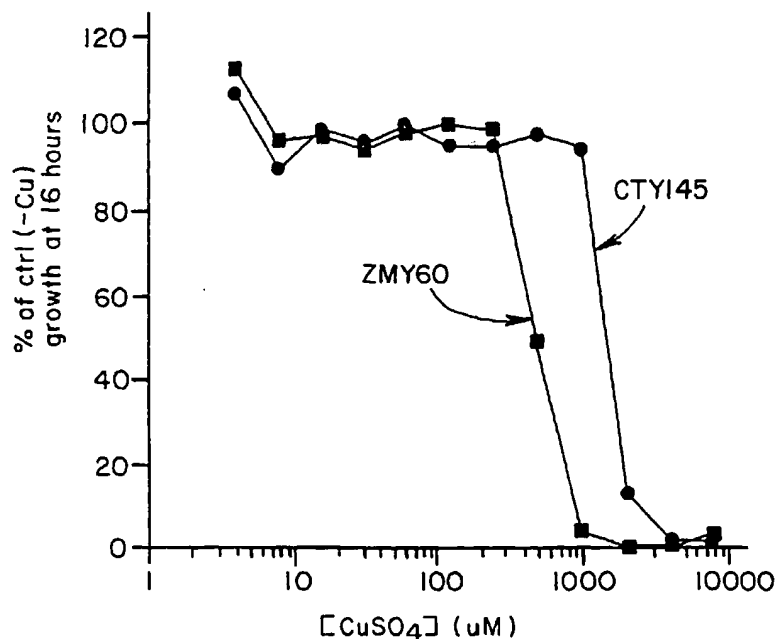
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ance Notes on Codes and Abbreviations" appearing at the begin-  
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(54) Title: REGULATED GENE EXPRESSION IN YEAST AND METHODS OF USE



(57) Abstract: The invention provides novel yeast cells comprising genes whose expression can be modulated by growth in the presence or absence of metal ions, methods for making such yeast cells, and methods of using such yeast cells for determining the requirement for expression of particular genes for the growth or viability of the yeast cells. The invention also provides methods of using such yeast cells in the isolation, screening and analysis of candidate antifungal compounds.

## REGULATED GENE EXPRESSION IN YEAST AND METHODS OF USE

5 This application is a continuation-in-part of U.S. Patent Application Serial No. 09/404,066, filed September 23, 1999, which is a division of U.S. Patent Application No. 09/138,024, filed August 21, 1998, now U.S. Patent No. 6,004,779, which claims priority under 35 U.S.C. § 119 from U.S. Provisional Patent Application 60/056,719, filed August 22, 1997, each of which are hereby incorporated by reference in its entirety.

### 10 Field of the Invention

The present invention relates to methods and compositions for regulated expression of specific genes in *Saccharomyces cerevisiae*. The invention can be used to identify and clone genes of interest and to identify antifungal agents using high-throughput screening techniques. The invention also relates to the use of the  
15 *Saccharomyces cerevisiae* strains of the invention in the isolation and analysis of antifungal agents.

### Background of Invention

The ability to regulate the expression of particular genes of interest is  
20 important for many purposes, including, for example, (i) investigation of the biological function of a particular gene product; (ii) design of variants of the gene product that are tailored for different ends; and (iii) identification of agents that influence the activity of the gene product, including, e.g., inhibitors or activators. The ease of performing genetic and molecular manipulations in *S. cerevisiae* has made it an extremely useful  
25 experimental organism for regulated expression of recombinant genes. However, many gene expression systems based on *S. cerevisiae* are limited in their applicability by (i) the degree of regulation that can be achieved, i.e., the extent to which genes can be turned

on and off, as well as the timing of these events; (ii) the relative stability of certain gene products, which makes it difficult to quickly deplete the cell of a gene product; and (iii) potential metabolic side effects of the procedures used to trigger or initiate changes in gene expression.

5                   Thus, there is a need in the art for *S. cerevisiae* expression systems in which gene expression can be tightly and efficiently regulated, with respect to both transcription of the gene and accumulation of the protein product.

#### **Summary of the Invention**

10                   The present invention encompasses yeast strains in which expression of a particular protein (the "subject" protein) can be tightly regulated. The invention provides *Saccharomyces cerevisiae* cells in which expression of the subject protein can be repressed by exogenous metal. These cells comprise, for example:

(i)           a first gene encoding a transcriptional repressor protein, the  
15           expression of which has been placed under the control of a metal ion-responsive element, wherein expression of the repressor protein is stimulated by the addition of a metal ion to the growth medium of the cells;

(ii)          a second gene encoding a subject protein, wherein  
                expression of the subject protein is controlled by a promoter, the activity of which is  
20           inhibited by said repressor protein; and

(iii)         a third gene encoding a biomineralization protein, wherein  
                the third gene is inactivated and wherein inactivation of the third gene enhances the  
transcriptional response of the metal-responsive element to added metal ions.

                In a preferred embodiment, the first gene is ROX1; the second gene is  
25           controlled by an ANB1 promoter; and the third gene is SLF1.

                In another embodiment, the yeast cell comprises a fourth gene encoding

a protein that targets ubiquitin-containing polypeptides for degradation, where the fourth gene is placed under the control of a metal ion-responsive element. In a preferred embodiment, the fourth gene is the UBR1 gene.

The invention further comprises yeast cells in which expression of the subject protein is stimulated by exogenous metal ions. These cells comprise:

- (i) a first gene encoding a subject protein, wherein expression of the gene encoding the subject protein is under the control of a metal ion-responsive element and is stimulated by the addition of a metal ion to the growth medium of the cells; and
- (ii) a second gene encoding a biomineralization protein, wherein the second gene is inactivated and wherein inactivation of the second gene enhances the transcriptional response of the metal-responsive element to added metal ions.

In a preferred embodiment, the metal-responsive element is the Sc3451 promoter and the second gene is SLF1.

In another aspect, the invention relates to a method for the introduction of a subject gene under the control a predetermined promoter DNA sequence into a yeast cell genome, comprising the steps of providing a shuffled gene fragment, where the fragment comprises a restriction enzyme cleavage sequence, ligating the shuffled gene fragment into a vector, where the ligation results in the shuffled gene fragment being operably linked to a predetermined transcriptional control DNA sequence, cutting the vector with a restriction enzyme specific for the restriction enzyme cleavage sequence to yield a linearized vector, and transforming a yeast cell with the linearized vector.

The invention also provides methods for repressing or activating expression of a gene encoding a subject protein in *S. cerevisiae* to a predetermined level, comprising culturing the strains described above in the presence of metal, wherein the

metal is present at sufficient concentration to activate the metal-responsive element so as to achieve the predetermined level of repression or activation of the gene.

In a further embodiment, the invention also encompasses methods for generating the yeast strains of the invention comprising:

- 5 (a) generating a yeast cell comprising
- (i) a first gene encoding a transcriptional repressor protein whose expression is under the control of a metal ion-responsive element, wherein expression of said first gene encoding said repressor protein is stimulated by the addition of a metal ion to growth medium of said yeast cell;
  - 10 (ii) a second gene encoding a subject protein, wherein expression of said second gene encoding said subject protein is controlled by a transcriptional control sequence whose activity is inhibited by said repressor protein; and
  - (iii) a third gene encoding a biomineralization protein, wherein said third gene is inactivated and wherein inactivation of said third gene enhances  
15 transcriptional response of said metal ion-responsive element to metal ions in said growth medium of said yeast cell;
- (b) culturing the yeast cell in a growth medium comprising metal ions, wherein said metal ions are present in sufficient concentration to activate said metal ion-responsive element to a level which will result in said predetermined level of repression  
20 of expression of said subject gene;
- (c) assessing whether the rapid depletion of the second gene from the yeast cell leads to inhibition of cell growth or cell death.

In a preferred embodiment the identifies the target gene as an essential target gene.

In another embodiment the invention is directed to methods of screening a candidate antifungal compound for interaction with an essential target gene comprising:

(a) generating a regulated yeast strain comprising a regulated essential target gene of the invention;

5 (b) establishing a concentration of metal ion at which the growth or viability of the regulated yeast strain ceases;

(c) generating a serial dilution of metal ion in yeast growth media;

(d) culturing the regulated yeast strain in the serially diluted growth media, wherein the serial dilution leads to a dose-dependent modulation of expression of the regulated essential target yeast gene product;

10

(e) screening the serially diluted cultures for altered sensitivity of the strain to the candidate antifungal compound;

(f) determining the metal ion concentration present in a culture demonstrating altered sensitivity to the candidate antifungal compound;

15 (g) comparing the metal ion concentration of step (b) with the metal ion concentration of the culture determined in step (f), and identifying a candidate antifungal compound for which a lower concentration of metal ion is required to eliminate growth or viability in step (f) as compared to step (b).

The method of screening may screen a single candidate or a plurality of candidate antifungal compounds. When a plurality of candidates screened, the screen is selected from the group consisting of screening together in a single assay and screening individually using multiple simultaneous individual detecting steps.

20

The invention also encompasses methods of rapidly cloning a DNA complementary to an essential target gene comprising:

(a) generating a regulated yeast strain comprising a regulated essential target gene of the invention;

(b) establishing a concentration of metal ion at which the growth or viability of the regulated yeast strain ceases;

5 (c) transforming the regulated yeast strain with a DNA to be tested for complementation;

(d) culturing the transformed regulated yeast strain in growth media containing a concentration of metal ion as established in step (b);

(e) determining the ability of the DNA to complement the regulated  
10 essential target gene, wherein growth or viability of the regulated yeast strain establishes complementation; and

(f) cloning the complementary DNA.

In a further embodiment, the DNA is selected from the group consisting of genes from another organism, mutant DNA and DNA fragments which can be  
15 generated from either genomic or cDNA libraries. In a further embodiment, the DNA is selected from an organism selected from the group consisting of human, mouse, mammal, drosophila and mycete.

The invention further encompasses methods of determining the antifungal effect of an antifungal compound comprising:

20 (a) generating a regulated yeast strain comprising a regulated essential target gene of the invention;

(b) establishing a concentration of metal ion at which the growth or viability of the regulated yeast strain ceases;

(c) culturing the regulated yeast strain in growth media containing the

concentration of metal ion as established in step (b);

(d) determining the phenotype associated with the culture of step (c) that is depleted of the essential target gene;

(e) culturing a yeast strain in growth media with a candidate antifungal compound;

(f) determining the phenotype associated with the culture of step (e) that is treated with the candidate antifungal compound;

(g) comparing the phenotypes determined in steps (d) and (f) to determine the antifungal effect of the antifungal compound.

In a further embodiment, the phenotypes are determined by

(i) incubating the cultures with radio-labeled macromolecular building-blocks;

(ii) establishing a level of incorporation of the radio-labeled macromolecular building blocks for each culture; and

(iii) analyzing the macromolecular products generated in each culture.

#### **Brief Description of the Drawings**

Figure 1 is a graphic illustration of the growth of yeast strains CTY145 and ZMY60 in increasing concentrations of copper sulfate. CTY145 is four-fold more tolerant to copper than ZMY60.

Figures 2A-C are schematic illustrations of the single- or double-round PCR strategy of the present invention that is used to construct a copper-inducible promoter element for any gene of interest. For single round PCR, primer pairs 1 and 2a or 2b are used to produce the transforming DNA. For the double-round PCR, primer



pairs 2a or 2b are used with additional primers corresponding to sequences located 400-1000 bp upstream or downstream of the ATG start site to prepare long primers which are then used in a second round of PCR to produce transforming DNA.

Figure 3 is a schematic illustration of a PCR primer design strategy that can be used to render a gene encoding a protein of interest repressible by LexA. The upper panel shows the structure of the promoter complex that can be inserted upstream of the gene of interest. The middle panel shows the location of the sequences corresponding to the PCR primers that can be utilized. The lower panel shows the resulting promoter cassette fused to the gene of interest which can be introduced into yeast.

Figures 4A-D illustrate the nucleotide sequence of the ZM195 plasmid (SEQ ID NO:20).

Figures 5A-D illustrate the nucleotide sequence of the ZM197 plasmid (SEQ ID NO:21).

Figure 6 is a schematic illustration of the PCR strategy used to generate shuffled genes for transformation into *S. cerevisiae*.

Figure 7 is a schematic illustration of the transformation mechanism by which shuffled genes are introduced into *S. cerevisiae*.

Figure 8 is a restriction map of the pCU19Srf vector (SEQ ID NO:22), showing the unique restriction enzyme cutting sites.

Figure 9 is a photograph of cell filtrates demonstrating the effect of deleting the SLF1 gene on biomineralization by yeast.

Figure 10 is a graph demonstrating the effect of copper sulfate on the growth of yeast strains which express and do not express the SLF1 gene.

Figure 11 is a graph showing growth curves for control and recombinant yeast strains which were grown in the presence of copper sulfate.

Figure 12 is an schematic example of a drug sensitivity dilution assay.

Figure 13 is an example of the set up for a drug sensitivity plate assay.

5        Figure 14 is a photograph showing the effect of copper sulfate on the growth of yeast strains that express wild type, mutated and Cu-regulated forms of the BOS1 gene.

Figure 15 provides Northern blot analysis showing the effect of copper sulfate on tRNA synthesis in yeast strains with the RPC34 and TAF145 Cu-regulated.

10        Figure 16 is a series of photographs of microtiter plates demonstrating the effect of increasing concentrations of copper sulfate on yeast strain sensitivity to tunicamycin in strains which express Cu-regulated ALG-7 (panel a) and CHS3 (panel b) and in wild type (panel c).

15        Figure 17 is a series of photographs demonstrating the effect of increasing concentrations of copper sulfate on yeast strain sensitivity to various antifungal compounds.

#### **Detailed Description of the Invention**

20        All patents, patent applications, publications and other materials cited herein are hereby incorporated by reference in their entirety. In the case of inconsistencies, the present description, including definitions, is intended to control.

As used herein, the term "transcriptional repressor protein" refers to a protein which either binds directly to a transcriptional control sequence or which binds

in association with other proteins or cofactors to a transcriptional control sequence, resulting in the repression of transcription of the protein encoding nucleotide sequence or sequences to which the transcriptional control sequence is operably linked.

As used herein, the term "transcriptional control sequence" refers to DNA sequences, such as initiator sequences, enhancer sequences, and promoter sequences, which induce, repress, or otherwise control the transcription of protein encoding nucleic acid sequences to which they are operably linked.

As used herein, the term "metal-ion responsive element" refers to a transcriptional control sequence which is activated when in the presence of an appropriate concentration of metal ions.

As used herein, the term "inactivated", when referring to a gene, means that the gene cannot be transcribed, either due to deletion of the gene from a genome or by disruption of its coding or regulatory sequences.

As used herein, the term "biomineralization protein" refers to a protein that promotes or catalyzes the conversion of ionic copper to a form insoluble in water, such as CuS.

As used herein, the term "shuffled gene fragment" refers to the nucleotide sequence around the ATG initiation codon of a gene, from about 400 nucleotides upstream of (i.e., 5' to) the ATG initiation codon of the gene to about 400 protein coding nucleotides downstream of (i.e., 3' to) the ATG initiation codon of the gene, wherein the orientation of the upstream and downstream sequences have been changed such that the ATG initiation codon and the approximately 400 downstream protein coding nucleotides that follow the ATG codon in the wild-type gene are upstream to the approximately 400 noncoding nucleotides normally found adjacent and upstream of the ATG initiation

codon. The shuffled gene fragment will typically contain a restriction enzyme cleavage sequence between the rearranged coding and noncoding nucleotide sequences.

As used herein, the term "restriction enzyme cleavage sequence" refers to a specific nucleotide sequence which is specifically recognized and cleaved by one or more restriction endonuclease enzymes.

As used herein, the term "operably linked" refers to the covalent attachment, typically of a transcriptional control sequence to a protein encoding nucleotide sequence, such that transcription of the protein encoding nucleotide sequence is regulated or controlled by the transcriptional control sequence.

As used herein, the term "linearized vector" refers to the cleavage product of circular double stranded DNA molecule, or vector, which has been cleaved at a single site, yielding a linear double stranded DNA molecule.

As used herein, "inhibition" refers to a reduction in the parameter being measured, whether it be fungal growth, DNA transcription, protein synthesis, etc.. The amount of such reduction is measured relative to a standard (control). Because of the multiple interactions of many *Saccharomyces* proteins in cell division, growth and cell cycle regulation, the particular target gene product for detection may vary with respect to the particular screening assay employed.

As used herein, "reduction" is defined as a decrease of at least 25% relative to a control, preferably of at least 50%, and most preferably of at least 75%.

As used herein, "growth" refers to the normal growth pattern of *S. cerevisiae*, i.e., to a cell doubling time of 60-90 minutes during the log phase of growth. In rich media, wild-type *S. cerevisiae* strains have a doubling time of 90 minutes. Growth of the cells may be measured by following the optical density of cells in liquid media.

An increasing optical density indicates growth. Growth can also be measured by colony formation from single cells on solid media plates.

As used herein, "viability" refers to the ability of the *S. cerevisiae* cells to resume growth following a treatment of the cells which results in cessation of growth. Examples of such treatments resulting in cessation of growth include, but are not limited to, transient inactivation of a gene product required for growth or treatment with an antifungal drug. One typical means by which viability is measured is by testing the ability of cells to form colonies on solid media plates following removal of the treatment which resulted in a cessation of growth. Cells that fail to form colonies are considered inviable.

As used herein, "cidal" is defined as a rapid loss in viability. Rapid is defined as a population of cells losing viability with a measured half-life of at least about 2 hours or less.

As used herein, "candidate inhibitor" is any compound with a potential to inhibit *Saccharomyces cerevisiae* growth or viability and may be used interchangeably with the term "candidate antifungal".

The present invention encompasses methods and compositions for regulating the expression of a gene of interest in *Saccharomyces cerevisiae*. The invention provides recombinant yeast strains which comprise:

- (i) a gene encoding a transcriptional repressor protein, the expression of which gene has been placed under the control of a metal ion-responsive element, so that expression of the gene encoding the repressor protein is stimulated by the addition of a metal ion to the growth medium of the cells;
- (ii) a gene encoding a protein of interest, the expression of which gene is inhibited by the repressor protein described in (i); and
- (iii) one or more genes involved in metal ion metabolism that have either been inactivated or overexpressed, depending on the gene, to enhance the

transcriptional response to added metal ion.

In the above yeast cells (a large number of such clonal cells being collectively designated "repressing strains"), the gene of interest is expressed in the absence of added metal ion. When it is desired to decrease or eliminate expression of the gene of interest, metal ions are added to the medium, which stimulates expression of the repressor to a degree that is dependent upon the concentration of added metal ions and represses transcription of the gene of interest.

The invention also encompasses yeast cells (a large number of such clonal cells being collectively designated "inducing strains") in which: (i) the gene of interest is operably linked to a metal ion-inducible transcriptional control sequence, so that expression of the gene of interest is directly stimulated by addition of metal ions to the medium; and (ii) one or more genes involved in metal ion metabolism that have either been inactivated or overexpressed, depending on the gene, to enhance the transcriptional response to added metal ion.

The choice of yeast strain in which the above manipulations are performed is important in practicing the invention. Suitable strains are those that tolerate the addition of metal ions to their culture medium at a sufficient concentration, and for a sufficient time period, to allow maximal expression of metal-inducible genes while maintaining cell viability and metabolism. Preferably, the growth rate of the strain should remain substantially unaffected for at least about 16 h after the addition of at least 750 mM copper sulfate, most preferably at least 1 mM copper sulfate. In addition, the strain should grow well and should be auxotrophic for common nutrients such as histidine, leucine, and uracil, to enable the use of, e.g., HIS3, LEU2, and URA3 as markers for genetic insertions. Suitable yeast strains include without limitation CTY145

(ATCC #74466) and S288C (ATCC #26108).

In some embodiments, the repressing strains of the invention further comprise a gene encoding a protein that targets ubiquitin-containing polypeptides for degradation via the ubiquitin degradation pathway, which, similar to the repressor gene,  
5 is expressed under the control of a metal ion-responsive regulatory element. In these embodiments, the gene of interest is expressed as a fusion protein, which contains at its amino terminus additional amino acids comprising a sequence that targets the polypeptide for the ubiquitin degradation pathway. In this manner, addition of metal ions to the medium also stimulates degradation of the protein of interest by the ubiquitin  
10 pathway, thereby depleting the protein from the cell. It will be understood, however, that some proteins of interest cannot be expressed in functional form as ubiquitin-targetable fusion proteins. Furthermore, overexpression of a ubiquitin-pathway gene may exert pleiotropic and potentially deleterious effects. Accordingly, the invention also encompasses repressing strains that do not overexpress a ubiquitin pathway protein and  
15 in which the gene of interest is not expressed as a fusion protein.

In practicing the invention, any metal ion-responsive transcriptional control element may be used, including without limitation DNA sequences comprising the binding site for the ACE1 protein, which has been identified as the sequence spanning nucleotides -105 to -148 of the CUP1 (metallothionein) promoter (Huitbregtse et al.,  
20 *Proc.Natl.Acad.Sci.USA* 86:65, 1989). Metal ion-responsive elements may be used singly or in tandem repeats, in direct or reverse orientation relative to a transcription start site, and may be combined with any compatible promoter such as, e.g., the HIS3 promoter. In conjunction with these elements, any suitable metal ion may be used to stimulate expression, including without limitation Ag, Cu, Cd, Ni, Zn, and Fe ions.

Suitable repressor proteins for use in the invention include without limitation ROX1, a heme-induced repressor of hypoxic genes (Genbank accession number #X60458) (Deckert et al., *Genetics* **139**:1149, 1995), LexA-CYC8 fusion proteins and LexA-TUP1 fusion proteins (Redd et al., *Cell* **78**:709, 1992). It will be understood that the choice of promoter sequences to be placed upstream of the gene of interest will be determined by the particular repressor used. For example, when ROX1 is the repressor, the promoters directing expression of the gene of interest may be derived from, e.g., the ANB1, HEM13, ERG11, or OLE1 genes. The sequences of these genes are disclosed under the following Genbank accession numbers: #M23440 (ANB1); #S81592 (HEM13); #U10555, U00093 (ERG11); and #U42698, #J05676 (OLE1). When the repressor contains bacterial LexA domains, the promoters directing the expression of the gene of interest may comprise sequences derived from the LexA operator. The sequence of the LexA operator is 5'-TACTGATGTACATACAGTA-3' (Tzamarias et al., *Nature* **369**:758, 1994) (SEQ ID NO:1); a synthetic LexA operator may also be employed, comprising the sequence:

5'-TCGAGTACTGTATGTACATACAGTACCATGACATACATGTATGTCATGAGCT-3'  
(U.S. Patent No. 4,833,080) (SEQ ID NO:2).

The genes involved in metal ion metabolism that may be inactivated to form the yeast strains of the present invention include without limitation SLF1, which is involved in the biomineralization pathway of copper (Genbank accession number U30375) (Yu et al., *Mol. Cell. Biol.* **16**:2464, 1996). In the case of SLF1, inactivation of the gene slows the depletion of copper from the growth medium and thereby enhances the transcriptional response of the repressor-encoding gene to the added copper ions. The result is an increase in the time period in which a consistent copper regulation of



gene expression can be maintained. Alternatively, genes encoding proteins such as, e.g., CTR1 (a metal ion transporter) can be overexpressed to increase the sensitivity of the transcriptional apparatus to the added metal ion (Dancis et al., *J. Biol. Chem.* **269**:25660, 1994).

5                   In the embodiments in which a ubiquitin-pathway protein is expressed under metal ion control, any ubiquitin-pathway protein may be expressed that will stimulate the degradation of an appropriately amino terminal tagged protein of interest. In one embodiment, the ubiquitin pathway protein that is linked to a metal ion-responsive element is UBR1 and the amino terminal tag is a hybrid sequence comprising, in amino-  
10   terminal-to-carboxyl terminal direction, ubiquitin and a 31-amino acid segment of the lac repressor protein (LacI), and may additionally include one or more epitope tags (Park et al., *Proc.Natl.Acad.Sci.USA* **89**:1249, 1992). In this embodiment, the hybrid protein (containing at its carboxyl terminus the protein of interest) is rapidly de-ubiquinated by yeast enzymes, and the resulting hybrid protein (containing an arginine residue at its  
15   amino terminus) is re-ubiquinated by the UBR1 protein (in the presence of a metal ion) and targeted for degradation.

Moqtaderi et. al., *Nature* **383**:188, 1996, disclose a haploid yeast strain (ZMY60) carrying integrated copies of the ROX1 and UBR1 genes which were placed under the control of the ACE1 promoter. Into this genetic background, a plasmid  
20   containing the ANB1 promoter driving expression of an in-frame fusion of ubiquitin, arginine, lacI, hemagglutinin epitopes and the full length gene of interest was introduced. Addition of 500 mM cupric sulfate (CuSO<sub>4</sub>) to the medium resulted in the repression of transcription of the gene of interest by ROX1 and rapid degradation of the ubiquitin-tagged protein. However, this strain is relatively genetically unstable resulting in

frequent reversion to a copper-insensitive phenotype, is highly sensitive to the toxic effects of copper ions at concentrations above 250 mM, and responds to copper ions for a relatively short time (in part, due to depletion of copper ions from the medium by biomineralization). The yeast strains of the present invention, by contrast, tolerate  
5 concentrations of copper ions of 1 mM or greater for extended periods of time. Furthermore, the yeast strains of the present invention exhibit more stable phenotypes, due to the use of methods which employ double-crossover events for integration of engineered genes into the yeast genome (see, e.g., Examples 3 and 4 below).

In one set of embodiments, the invention provides a CTY145-based yeast  
10 strain in which: (i) the native ROX1 gene promoter has been replaced with a promoter comprising a hybrid HIS3 promoter-ACE1 binding site; (ii) the native SLF1 gene has been deleted; and (iii) the gene of interest is controlled by an ANB1 promoter. Features (i)-(iii) are preferably achieved using a double-crossover strategy. In an alternate embodiment, the CTY145 strain has been modified as in (i) and (ii) above, and, in  
15 addition, (iii) the native UBR1 gene promoter has been replaced with a promoter comprising a hybrid HIS3 promoter-ACE1 binding site; and (iv) a sequence which comprises an ANB1 promoter followed by a sequence encoding a hybrid polypeptide comprising ubiquitin, a LacI fragment, and an epitope tag is fused to the 5' end of the protein-coding sequence of a gene of interest.

20 In another set of embodiments, the invention provides a CTY145-based yeast strain in which: (i) a gene has been introduced comprising a hybrid HIS3 promoter-ACE1 binding site placed upstream of sequences encoding a CYC8-LexA fusion protein; (ii) the native SLF1 gene has been deleted; and (iii) a gene of interest is controlled by a promoter comprising a LexA operator. Features (i)-(iii) are preferably achieved using a

double-crossover strategy. In an alternate embodiment, the CTY145 strain has been modified as in (i) and (ii) above, and, in addition, (iii) the native UBR1 gene promoter has been replaced with a promoter comprising a hybrid HIS3 promoter-ACE1 binding site; and (iv) a sequence which comprises a LexA operator-containing promoter followed  
5 by a sequence encoding a hybrid polypeptide comprising ubiquitin, a LacI fragment, and an epitope tag is fused to the 5' end of the protein-coding sequence of a gene of interest.

In another set of embodiments, the invention provides a CTY145-based yeast strain in which (i) the gene of interest is controlled by the Sc3451 promoter and (ii) the native SLF1 gene has been deleted. The Sc3451 promoter was constructed by cloning  
10 a n A C E 1 b i n d i n g s i t e ( 5 ' - TAAGTCTTTTTTGCTGGAACGGTTGAGCGGAAAAGACGCATC-3') (SEQ ID NO:3) upstream of the TATAA sequence at an EcoRI site in plasmid YIp55-Sc3370 (Struhl et al., *Mol. Cell Biol.* 7:104, 1987).

## 15 METHODS

In practicing the present invention, many conventional techniques in molecular biology, microbiology, and recombinant DNA, are used. Such techniques are well known and are explained fully in, for example, *Current Protocols in Molecular Biology*, Volumes I, II, and III, 1997 (F.M. Ausubel ed.); Sambrook et al., 1989,  
20 *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; *DNA Cloning: A Practical Approach*, Volumes I and II, 1985 (D.N. Glover ed.); *Oligonucleotide Synthesis*, 1984, (M.L. Gait ed.); *Nucleic Acid Hybridization*, 1985, (Hames and Higgins); *Transcription and Translation*, 1984 (Hames and Higgins eds.); *Animal Cell Culture*, 1986 (R.I. Freshney

ed.); *Immobilized Cells and Enzymes*, 1986 (IRL Press); Perbal, 1984, *A Practical Guide to Molecular Cloning*; the series, *Methods in Enzymology* (Academic Press, Inc.); and *Methods in Enzymology* Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively).

5           Insertion of nucleic acids (typically DNAs) comprising the sequences of the present invention into a vector is easily accomplished when the termini of both the DNAs and the vector comprise compatible restriction sites. If this cannot be done, it may be necessary to modify the termini of the DNAs and/or vector by digesting back single-stranded DNA overhangs generated by restriction endonuclease cleavage to produce blunt  
10 ends, or to achieve the same result by filling in the single-stranded termini with an appropriate DNA polymerase.

          Alternatively, any site desired may be produced, e.g., by ligating nucleotide sequences (linkers) onto the termini. Such linkers may comprise specific oligonucleotide sequences that define desired restriction sites. Restriction sites can also  
15 be generated by the use of the polymerase chain reaction (PCR). See, e.g., Saiki *et al.*, 1988, *Science* 239:48. The cleaved vector and the DNA fragments may also be modified if required by homopolymeric tailing.

          The nucleic acids may be isolated directly from cells. Alternatively, the polymerase chain reaction (PCR) method can be used to produce the nucleic acids of the  
20 invention, using either chemically synthesized strands or genomic material as templates. Primers used for PCR can be synthesized using the sequence information provided herein and can further be designed to introduce appropriate new restriction sites, if desirable, to facilitate incorporation into a given vector for recombinant expression.

          Methods for yeast transformation, integration of genes into the yeast

genome, and growth and selection of yeast strains are fully described in, e.g., *Current Protocols in Molecular Biology*, Vols. 1 and 2, Ausubel et al., eds., John Wiley & Sons, New York (1997). The use of URA3 for the production of multiply disrupted yeast strains is disclosed in Alani et al., *Genetics* **116**:541, 1987.

5                   A preferred method for the transformation of *S. cerevisiae* is as follows. Yeast strains are cultured overnight in YPD (yeast extract, peptone, dextrose) medium at about 30°C. The resulting culture is diluted to an  $A_{600}$  of about 0.2 in about 200 ml YPD medium and incubated at about 30°C until the  $A_{600}$  reaches approximately 0.8. The cells are pelleted by centrifugation and are washed in about 20 ml sterile water. The  
10                   pelleted yeast cells are then resuspended in about 10 ml TEL (10mM Tris pH 7.5, 1 mM EDTA, 0.1 M LiAcetate pH 7.5) buffer. The cells are pelleted by centrifugation and again resuspended in about 2 ml TEL. About 100 mg of well-sheared single stranded DNA and plasmid DNA are added to an eppendorf tube. To this tube is added about 100 ml of competent yeast cells, followed by mixing. To the cell/DNA mixture is added  
15                   about 0.8 ml of 40% PEG-3350 in TEL, followed by thorough mixing. This mixture is incubated for about 30 minutes at 30°C, followed by a heat shock for 20 minutes at 42°C. The mixture is centrifuged to remove the supernatant and pellet the cells. The yeast cell pellet is washed with about 1 ml TE, pelleted again by centrifugation, and then plated on selective media.

20                   A preferred method for the extraction of genomic DNA from *S. cerevisiae* for PCR is as follows. A 5 ml overnight yeast strain culture grown in YPD at 30°C is spun out by centrifugation and washed once in 1 ml Tris pH 7.5/1 mM EDTA (TE) buffer. The cells are pelleted again by centrifugation and resuspended in 0.2 ml Extraction Buffer (2% Triton X100, 1% SDS, 100 mM NaCl, 10mM Tris pH 7.5 and 1mM EDTA) plus

0.2 ml phenol/chloroform/isoamyl alcohol. About 0.3 g of acid washed glass beads are added. This mixture is vortexed (i.e., agitated vigorously) for 30 minutes. 0.2 ml TE buffer is then added. The mixture is centrifuged and the aqueous phase is removed. The DNA is precipitated from the aqueous phase with two volumes of ethanol. The precipitate  
5 is pelleted by microcentrifugation, and resuspended in 50 µl TE plus 5 mg/ml RnaseA enzyme. The resulting preparation can be diluted to a desired concentration, or used directly for PCR reactions.

For assaying the effects of copper ions on recombinant yeast strains, wild type and recombinant strains are grown in 5 ml of CSM media on a roller drum  
10 incubation apparatus at 30°C for 18 to 20 hours. Cultures are diluted to an  $A_{600}$  (absorbance of light at 600 nm) of about 0.02 in 5 ml CSM media without or with various concentrations of  $\text{CuSO}_4$  (10 mM, 50 mM, 100 mM, 250 mM, 1mM and 2 mM) for 18 to 20 hours. The  $A_{600}$  of the various samples is read and recorded.

Yeast strains are tested by a time course in the presence and absence of  
15 copper to determine if the depletion of target gene product is fungistatic (i.e., inhibitory to growth) or fungicidal (i.e., yeast killing). Cultures are started from a single yeast colony in CSM media (5 ml) and grown at 30°C for 18 -20 hours in a roller drum. Cultures are diluted in fresh media to a final volume of 10 ml at an  $A_{600}$  of about 0.25 and allowed to grow at 30°C for 1 hour. The cultures are split into two aliquots and 1 mM  $\text{CuSO}_4$  is  
20 added to one of the aliquots. A sample of 300 µl is immediately taken from each culture aliquot as the zero time point. Other similar samples are taken at 1, 3, 5, 7 and 24 hours after  $\text{CuSO}_4$  addition. Alternatively, the cultures can be diluted to an  $A_{600}$  of about 0.1 and allowed to grow for 3 hours, at which time the cultures are diluted again to an  $A_{600}$  of about 0.02, after which 1 mM  $\text{CuSO}_4$  is added. To measure the absorbance of a yeast

culture, typically two-hundred microliters of each sample is taken and added to a 96-well flat bottom polystyrene plate, which is then inserted into a plate reader where the absorbance at 595 nm is measured. A growth curve can be generated from these readings.

5                   When plating cells on YPD medium for analysis of CFU number, typically 100 ml of each sample is serially diluted in 900 ml sterile water. Plating dilutions for yeast cultured without copper ions and for wild type yeast cultured in the presence of copper ions are from  $10^{-3}$  to  $10^{-6}$ . Plating dilutions for time points 0, 1, 3, 5, and 7 hrs for recombinant yeast cultured in the presence of copper ions range from  $10^{-2}$  to  $10^{-5}$ . Plating  
10 dilutions for any 24 hour time points for yeast cultured in the presence of copper ions ranges from undiluted to  $10^{-2}$ .

Typically, about one-hundred microliters of each dilution is plated on YPD agar plates and incubated at 30°C for 48 hrs. Colonies are counted and recorded. Calculations are made to convert colony counts to CFU/ml of original culture medium.

15

#### APPLICATIONS

The yeast strains of the present invention find use for:

(i)     *Rapid and efficient determination of whether a particular gene of interest can serve as a potential target for discovery of antifungal drugs.* The present  
20 invention encompasses methods of identifying target genes useful in the discovery of antifungal agents. Identification of potential targets is carried out by assessing whether the rapid depletion from yeast cells of a particular gene product (using the "repressing strains" described above) leads to inhibition or slowing of cell growth, or cell death. Since the most effective and preferred antifungal drugs are those whose effect is rapidly  
25 fungicidal, a gene product whose depletion leads to cell death is a preferred potential

target for a candidate inhibitor of fungal growth, *i.e.* an antifungal drug. Because the degree of the reduction in the amount of the gene product can be controlled by the concentration of metal added, it is further possible to determine the degree of reduction of the gene product necessary to cause cell death.

- 5                    Various methods can be used to determine whether the product of a gene is essential to the survival of *S. cerevisiae* and thus essential to the establishment or maintenance of an infection. The identification of the essential character of a gene provides additional information regarding its function and allows selection of genes for which the product constitutes a target of interest for an antifungal agent or substance.
- 10                   Examples of these methods are summarized briefly below. These methods are described in the following works, each of which are hereby incorporated by reference herein:
- Guthrie C. and Fink G.R. eds. Methods in Enzymology, Vol. 194, 1991, 'Guide to Yeast Genetics and Molecular Biology', Academic Press Inc.
  - Rose A.H., A.E. Wheals and J.S. Harrison eds. The Yeasts, Vol. 6, 1995, 'Yeast
  - 15                   Genetics', Academic Press Inc.
  - Ausubel F. et al. eds. Short Protocols in Molecular Biology, 1995, Wiley.
  - Brown A.J.P. and Tuite M.F. (eds) Methods in Microbiology, Vol. 26, 1998, 'Yeast Gene Analysis' Academic Press Inc.

                    Depending on the circumstances, one of the methods described may be

20                   used, depending on the desired result. In particular, it is possible to proceed by a method of either direct inactivation of the gene or transitory inactivation of the gene. In the yeast *S. cerevisiae*, the method used most generally comprises inactivation of the gene of interest at its site within the chromosome of the yeast. The wild type allele is inactivated by insertion of a genetic marker (for example a gene for auxotrophy or a resistance



marker). This insertion is in general obtained by the method of gene conversion with the aid of linear deletion cassettes prepared by known methods, as described in Guthrie C. and Fink G.R. eds. Methods in Enzymology, or in Gultner *et al. Nucleic Acid Research*, 1996, 24: 2519-2524.

5                   The method of the invention is directed to the isolation of an *S. cerevisiae* strain where the cidal target gene is under the regulation of copper as described above. The preferred copper regulated strains of the invention are those with copper regulated essential proteins which are required for growth and/or viability. In carrying out the method of the invention, an *S. cerevisiae* strain in which expression of a particular gene  
10                   can be tightly regulated by copper is generated as described above to be used in the methods as described below.

(ii)       *Identification of target gene products whose rapid depletion leads to increased sensitivity to known antifungal drugs and candidate antifungal compounds.*  
The present invention encompasses methods of identifying target gene products which,  
15                   when decreased, provide for increased sensitivity to known antifungal drugs or, more importantly, candidate antifungal compounds isolated through various screening methods. Examples of known antifungal agents include amphotericin B and other polyene macrolide compounds like nystatin; flucytosine; ketoconazole, fluconazole, itraconazole and other triazoles. It would be highly advantageous however to isolate new  
20                   antifungal compounds which attack fungi at one of a number of essential targets.

It has been shown that decreased gene dosage of a drug target results in increased sensitivity of the cell to the drug (Giaever *et al.*, Nature Genetics, 21:(3) 278-283, 1999). Giaever, *et al.* demonstrated this using heterozygous diploid strains of *S. cerevisiae* (one of two gene copies deleted). A similar approach can be used with the

engineered copper strains of the invention. Increasing concentrations of copper in the growth media will lead to decreased gene expression which results in increased sensitivity to compounds that inhibit the activity of the gene product in question. In effect, there is a synergy between copper and certain compounds. Compounds that do not display such synergy are less likely to be hitting the target of interest.

According to the invention, an engineered yeast strain of the invention, where the copper regulated gene has been shown to be an essential target, is used to evaluate and characterize the mechanism of action of such antifungal drugs and candidate antifungal compounds. Alteration of copper ion concentrations in the growth media of Cu-regulated strains leads to a dose-dependent modulation of the expression of the yeast gene product of interest. Altered sensitivity of these strains to a particular compound suggests that the gene is involved in mediating the action of the known antifungal drug, or compound of interest. Comparatively, compounds that do not demonstrate altered sensitivity are unlikely to produce antifungal effects through interaction with the modulated *i.e.* copper regulated gene product. Thus, the engineered yeast strains of the invention provide a tool for screening antifungal compounds in order to characterize the activity and gene product with which the compounds interact. This phenomenon also provides for the establishment and identification of potential synergies between known drugs and newly discovered antifungal compounds.

In one embodiment, such drug sensitivity is analyzed utilizing the copper regulated strains of the invention according to the following non-limiting method. In a single 96 well assay plate, a serial dilution of copper sulfate is created from right to left. On the same plate a serial dilution of compound is created along the opposite axis to give the final compound and copper concentrations shown in Figure 12. Each well is

inoculated with  $10^3$  cells  $\text{ml}^{-1}$ . Growth is scored by eye after 48 hours incubation at  $37^\circ\text{C}$ .

The patterns of growth expected for each plate are shown in Figures 13a-c. As the copper concentration is increased, the level of target expressed is reduced. Consequently, inhibition of growth should occur at a lower compound concentration (Figure 13, panel

5 a). If the compound does not inhibit the target no effect on growth in the presence of copper will be seen (Figure 13, panel b). A wild type strain should be used as a control to demonstrate that the copper concentration alone is not making the cells more susceptible to the compound. (Figure 13, panel c).

(iii) *Rapid cloning of functionally complementary DNA fragments or*  
10 *functionally complementary genes from other organisms, including pathogenic fungi*  
*such as C. albicans and A. fumigatus.* The present invention encompasses methods of rapidly cloning complementary genes from other organisms, particularly other pathogenic fungi, mutant DNA and DNA fragments. According to the invention, the engineered yeast cells of the invention are used to evaluate and characterize the ability of a given  
15 gene, mutant DNA or DNA fragment to complement a metal ion-regulated protein. As described above, the engineered yeast cells of the invention provide for the generation of conditional mutant strains where alteration of metal ion concentrations in the growth media of such metal ion-regulated strains leads to a slowing of cell growth and/or cell death by depletion of a particular yeast gene product which is essential for growth and or  
20 life of the cells. Transformation of these conditional mutants with cDNA or genomic DNA libraries from other species allows for the selection and identification of functional homologs to the depleted *Sacchromyces* gene product.

This type of analysis can also be used to test which region or regions of a given target protein are essential for function. This is carried out by transforming

the conditional Cu-regulated strains with fragments of the disrupted genes, or DNA isolated from mutants with known disruptions in the gene of interest, in order to determine the regions functionally necessary for complementation.

Complementation analysis utilizing conditional mutants is well known in the field of yeast genetics. Indeed, complementation has been demonstrated for a number of genes. Defects in the following *S. cerevisiae* genes are known to be complemented by homologs from the indicated species:

RHO1	<i>C. albicans</i> , human, drosophila
URA3	<i>C. albicans</i> , <i>S. pombe</i> , <i>C. utilis</i> , mouse
10 CDC68	<i>K. lactis</i>
RPL35	<i>S. pombe</i>
RPB6	<i>S. pombe</i> , human, drosophila
SPT15	<i>C. albicans</i> , <i>S. pombe</i> , <i>A. nidulans</i>
CMD1	<i>S. pombe</i> , <i>K. lactis</i> , <i>Xenopus laevis</i> , chicken

15

Thus, in one embodiment, the invention is directed to a method of rapidly cloning functionally complementary genes from another organism. Complementation analysis may be carried out using a gene or DNA fragment isolated from any organism, including human, mouse, or other mammal; drosophila; and other mycete fungi. Examples of the other mycetes to be analyzed for functionally complementary genes include *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quillermondii*, *Candida glabrata*, *Candida lusitanae* or *Candida rugosa*, or also mycetes of the type *Aspergillus* or *Cryptococcus*, and in particular, for example, *Aspergillus fumigatus*, *Coccidioides immitis*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis* and *Sporothrix schenckii*, or also mycetes of the classes of *Phycomycetes* or *Eumycetes*, in particular the sub-classes of

25

*Basidiomycetes, Ascomycetes, Mehliscomycetales* (yeast) and *Plectascales, Gymnascales* (fungus of the skin and hair) or of the class of *Hyphomycetes*, in particular the sub-classes *Conidiosporales* and *Thallosporales*, and among these the following species: *Mucor*, *Rhizopus*, *Coccidioides*, *Paracoccidioides* (*Blastomyces*, *brasiliensis*), *Endomyces* 5 (*Blastomyces*), *Aspergillus*, *Menicilium*. (*Scopulariopsis*), *Trichophyton* (*Ctenomyces*), *Epidermophyton*, *Microsporon*, *Piedraia*, *Hormodendron*, *Phialophora*, *Sporotrichon*, *Cryptococcus*, *Candida*, *Geotrichum*, *Trichosporon* or also *Toropsulosis*.

Complementation methods are well known in the art. It is known that given an essential gene in a species, genes which are homologous or have the same 10 function in another species can be identified. The methods known to those of ordinary skill in the art can be used to identify a homolog to a gene studied in another species of mycete (so-called "orthologous" genes) or other organisms genes having the same function as the gene studied. Examples of methods are described in the following works which are hereby incorporated by reference herein:

- 15 - Sambrook et al. 1989, Molecular Cloning, Cold Spring Harbor Laboratory Press.  
- Ausubel F. et al. eds. Short Protocols in Molecular Biology, 1995, Wiley.  
- Guthrie C. and Fink G.R. eds. Methods in Enzymology, Vol. 194, 1991, 'Guide to Yeast Genetics and Molecular Biology', Academic Press Inc.

Such methods include screening for homology or gene complementation to genomic or 20 cDNA libraries of pathogenic mycetes, or PCR amplification of such library DNA using specific primers selected by virtue of their homology to the nucleotide sequence of interest.

In the present invention, the metal ion-regulated conditional mutants of *Saccharomyces cerevisiae* are used to rapidly determine if a particular gene, gene 25 fragment or DNA can complement and thus be easily selected and cloned. Using the strains of the invention, it is not necessary to carry out homology analysis as described previously. Using the strains of the invention, genes and DNA fragments can be screened rapidly by determining if they provide complementation of an essential gene.

Genomic DNA or cDNA libraries can be prepared by known methods and the polynucleotide fragments obtained are integrated into an expression vector, for example a vector such as pRS423 or its derivatives, which can be used both in the bacterium *E. coli* and in *S. cerevisiae*.

5 In one embodiment, an engineered *S. cerevisiae* strain of the invention in which an identified essential gene has been placed under metal ion regulation is transformed by a representative sample of a DNA or cDNA library corresponding to the organism being studied. Such methods can also be used in the analysis of complementation by a given DNA fragment or mutant DNA. As described above, when  
10 the yeast strains of the invention are cultured in the presence of metal ion, the promoter is repressed and the only yeasts that can survive are the ones that carry a recombinant vector containing a sequence which is functionally equivalent to, or complements, the regulated *S. cerevisiae* essential gene.

Following determination of complementation, the complementing gene  
15 or DNA sequence can be further characterized and sequenced by isolating the recombinant vector and sequencing it by known methods.

This type of study can be performed on various species: the genes which are functionally equivalent or homologous in sequence to an essential gene can be isolated in other mycetes, and in particular in the various mycetes which are pathogenic  
20 to humans which include, in particular, mycetes belonging to the classes *Zygomycetes*, *Basidiomycetes*, *Ascomycetes* and *Deuteromycetes*. More particularly, the mycetes will belong to the sub-classes *Candida* spp., in particular *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis* and *Candida krusei*. The mycetes will also belong to the sub-classes *Aspergillus fumigatus*, *Coccidioides immitis*,

*Cryptococcus neoformans*, *Histoplasma capsulatum*, *Blastomyces dermatidis*, *Paracoccidioides brasiliensis* and *Sporothrix schenckii*.

(iv) *Development of libraries of strains, each of which contains a different gene which is either positively or negatively regulated by metal ions.* The present invention encompasses the development of libraries of the metal ion-regulated strains of the invention. Such libraries are useful for identifying targets for antifungal drugs whose mechanism of action is unknown. For example, if stimulation or repression of expression of a particular gene leads to decreased and increased sensitivity, respectively, to a particular drug, then the gene is likely to be involved in mediating the *in vivo* action of the drug. Evaluating the effect of modulation of the gene product, by the presence or absence of metal ion, on cell physiology leads to understanding the physiological role of the gene product. Further, comparison of phenotypes associated with cells depleted of certain gene products should yield similar phenotypes in that compounds inhibit that gene product. Large strain libraries can easily be created since the entire *S. cerevisiae* genome has been sequenced and all open reading frames identified (Goffeau et al., *Science* **274**:563, 1996; Goffeau et al., *Nature* **387**:5, 1997). The majority of these open reading frames and corresponding encoded proteins have been characterized (Costanzo et al., *Nucleic Acids Res.* **28**:73, 2000; Ball et al., *Nucleic Acids Res.* **28**:77, 2000; Mewes et al., *Nucleic Acids Res.* **28**:37, 2000)

(v) *Development of high-throughput screening methodologies to detect antifungal compounds.* The present invention also encompasses methods wherein the engineered yeast cells of the invention are used to evaluate and characterize compounds as potential antifungal compounds. Alteration of the concentration of metal ions leads to a dose-dependent modulation of the expression of the regulated yeast gene

product. Altered sensitivity of these cells to a particular compound, suggests that the controlled gene product is likely to be involved in mediating the action of the compound and lead to the determination of that compound as a candidate inhibitor.

Such compounds may be found in, for example, natural product libraries, fermentation libraries (encompassing plants and microorganisms), combinatorial libraries, compound files, and synthetic compound libraries. For example, synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich Chemical Company, Inc. (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from, for example, Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondelle et al., *TibTech* **14**:60, 1996).

Thus, in a further embodiment of the invention, following the determination that a given *Saccharomyces cerevisiae* strain of the invention contains a metal ion-regulated cidal target, the strain may be used to isolate candidate inhibitors of fungal growth and/or infection. In carrying out the screening methods of the invention, which involve screening a plurality of candidate inhibitor compounds, *i.e.* candidate antifungal compounds, the plurality of candidate antifungal compounds may be screened together in a single assay or individually using multiple simultaneous individual detecting steps. The methods used to determine altered sensitivity to candidate antifungal compounds as described above are used in carrying out the screening of the plurality of candidates.

As noted above, a "candidate inhibitor," as used herein, is any compound



with a potential to inhibit the growth and viability of *Saccharomyces cerevisiae*, or other fungi. The methods of the invention are directed to the identification of candidate inhibitor compounds in a primary screen against the *Saccharomyces cerevisiae* metal ion-regulated strains of the invention.

5                   Candidate inhibitors are screened by measuring susceptibility of the *Saccharomyces cerevisiae* strains of the invention where an essential gene is under the control of metal ion. According to the methods of the invention, a plurality of candidate inhibitors are screened utilizing a given strain of the invention to determine if the cells are killed or grow slower in a lower concentration of metal ion. If a lower concentration  
10                   of metal ion is required to kill or slow growth, then the compound being screened is characterized as a potential antifungal.

#### Candidate Inhibitor Preparation:

                  Stock solutions and concentrations tested will vary from compound to  
15                   compound. In one non-limiting embodiment, stock solutions of 12.8 mg/ml in DMSO (Sigma D-8779) should be prepared. This will allow for a 128 ug/ml starting test concentration containing 1% DMSO. Stock solutions should be stored at -20°C and dilutions for antifungal testing should be freshly prepared before each assay.

                  For compounds of unknown activity or ones with MIC values of >4 ug/ml,  
20                   a range of concentrations from 128 ug/ml to 0.125 ug/ml should be used. More active compounds, such as Amphotericin B (Sigma A2411) and Itraconazole (Research Diagnostics Inc. cat# 30.211.44), require a lower range of concentrations (16 ug/ml to 0.016 ug/ml). Stock solutions of Amphotericin B and Itraconazole should be prepared at 1.6 mg/ml in DMSO. Amphotericin B is sold as a powder that is approximately 80%  
25                   Amphotericin B. Stock solutions should be made accordingly (2.0 mg of powder for a 1 ml solution of 1.6 mg/ml Amphotericin B).

Stock solutions of control compounds (1.6 mg/ml, Amphotericin B or Itraconazole) are initially diluted in medium to a concentration of 32 ug/ml while stock solutions of test compounds (typically 12.8 mg/ml) are diluted to 256 ug/ml. Both of these (control and test compounds) represent 1:50 dilutions. If a stock solution of a test compound is not at 12.8 mg/ml, the appropriate dilution must be calculated. Serial dilutions will be produced (see below) using these initial dilutions. Addition of cells to compound will produce an additional two-fold dilution.

Natural product extracts are tested at concentrations ranging from 200 to 204,800 fold dilution of the extract based upon the initial culture volume. The extract should first be diluted 100 fold then serial dilutions produced as directed below.

\* \* \* \* \*

Compounds that interact with the target gene of interest with have an effect on the concentration of metal ion required to kill or slow the growth of the strain of interest. Such compounds are considered for further development.

(vi) *Phenocopy control for drug treated cells.* The present invention also encompasses methods of determining the phenotype generated by a particular antifungal agent. Phenotypes associated with cells that are depleted of a specific gene product, should produce a similar phenotype to that of cells treated with a compound that specifically inhibits that protein as its target. If a metal ion repressed cell produces the same phenotype as a cell treated with a particular antifungal drug, it would suggest that the compound is producing its effect through interaction with the gene or gene product being evaluated. Indeed, if the phenotype of a compound treated strain is similar to or the same as the phenotype of the target gene, such compound can be associated with inactivation of the target gene or protein.

Phenotypes associated with decreased cell viability and/or growth can fall into many categories. Non-limiting examples of such categories may include: absence of DNA synthesis, absence of RNA synthesis, absence of transfer of RNA, absence of tRNA, absence of cell division, absence of cell cycling, absence of growth, and inability to make membrane. Non-limiting examples of essential cellular functions and associated genes are growth (BOS1); viability (RPL31); cell cycle control (CDC23); cell wall maintenance (GPL3); DNA synthesis (POL30); nuclear-cytoplasmic transport (NPL6); tRNA synthesis (RPC34); rRNA synthesis (RRN3); mRNA synthesis (SRB4); protein synthesis (NIP1); and RNA splicing (PRP21). References for some of these examples, as well as other examples of cellular functions and known mutations in *S. cerevisiae* are provided as follows:

**\* Protein transport from the endoplasmic reticulum (ER) to the Golgi complex**

**BOS1 mutants**

Wuestehube LJ, Duden R, Eun A, Hamamoto S, Korn P, Ram R, Schekman R. New mutants of *Saccharomyces cerevisiae* affected in the transport of proteins from the endoplasmic reticulum to the Golgi complex.

Genetics 1996 Feb;142(2):393-406

**\* DNA replication and repair**

**POL30 mutants**

Merrill BJ, Holm C. The RAD52 recombinational repair pathway is essential in pol30 (PCNA) mutants that accumulate small

single-stranded DNA fragments during DNA synthesis. Genetics 1998 Feb;148(2):611-24

**\* Protein translation**

**DED1 mutants**

Chuang RY, Weaver PL, Liu Z, Chang TH. Requirement of the DEAD-Box protein ded1p for messenger RNA translation. Science 1997 Mar

7;275(5305):1468-71.

**\* mRNA splicing**

PRP21 mutants

- Arenas JE, Abelson JN. The *Saccharomyces cerevisiae* PRP21 gene product is an integral component of the prespliceosome. Proc Natl Acad Sci U S A 1993 Jul 15;90(14):6771-6775.

**\* tRNA splicing**

TRL1 mutants

- Phizicky EM, Consaul SA, Nehrke KW, Abelson J. Yeast tRNA ligase mutants are nonviable and accumulate tRNA splicing intermediates. J Biol Chem 1992 Mar 5;267(7):4577-82

**\* rRNA synthesis**

RRN3 mutants

- Nogi Y, Vu L, Nomura M. An approach for isolation of mutants defective in 35S ribosomal RNA synthesis in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 1991 Aug 15;88(16):7026-30

**\* Long chain fatty acid synthesis**

- 20 ACC1 mutants

- Schneider R, Hitomi M, Ivessa AS, Fasch EV, Kohlwein SD, Tartakoff AM. A yeast acetyl coenzyme A carboxylase mutant links very-long-chain fatty acid synthesis to the structure and function of the nuclear membrane-pore complex. Mol Cell Biol 1996 Dec;16(12):7161-72

**\* Protein N-glycosylation**

ALG1mutants

- Benton BK, Plump SD, Roos J, Lennarz WJ, Cross FR. Over-expression of *S. cerevisiae* G1 cyclins restores the viability of alg1 N-glycosylation mutants. Curr Genet 1996 Jan;29(2):106-13

**\* Sphingolipid synthesis**

## AUR1 mutants

Nagiec MM, Nagiec EE, Baltisberger JA, Wells GB, Lester RL, Dickson RC. Sphingolipid synthesis as a target for antifungal drugs.

- 5 Complementation of the inositol phosphorylceramide synthase defect in a mutant strain of *Saccharomyces cerevisiae* by the AUR1 gene. J Biol Chem 1997 Apr 11;272(15):9809-17

## \* tRNA nucleotidyltransferase

## CCA1 mutants

- 10 Chen JY, Joyce PB, Wolfe CL, Steffen MC, Martin NC. Cytoplasmic and mitochondrial tRNA nucleotidyltransferase activities are derived from the same gene in the yeast *Saccharomyces cerevisiae*. J Biol Chem 1992 Jul 25;267(21):14879-83

## 15 \* Ergosterol biosynthesis

## ERG11 mutants

- Trocha PJ, Jasne SJ, Sprinson DB. Yeast mutants blocked in removing the lanosterol at C-14. Separation of sterols by high-pressure liquid chromatography. Biochemistry 1977 Oct 18;16(21):4721-6

20

## \* Polyadenylation

## FIP1 mutants

- Preker PJ, Lingner J, Minvielle-Sebastia L, Keller W. The FIP1 gene encodes a component of a yeast pre-mRNA polyadenylation factor that  
25 directly interacts with poly(A) polymerase. Cell 1995 May 5;81(3):379-89.

## \* Nuclear export of RNA

## GLE1 mutants

- 30 Murphy R, Wente SR. An RNA-export mediator with an essential nuclear export signal. Nature 1996 Sep 26;383(6598):357-60

\* rRNA processing

LCP5 mutants

Wiederkehr T, Pretot RF, Minvielle-Sebastia L. Synthetic lethal interactions with conditional poly(A) polymerase alleles identify

5 LCP5, a gene involved in 18S rRNA maturation. RNA 1998 Nov;4(11):1357-72

With the emergence of high throughput screening ("HTS") methodologies for identifying candidate antifungal compounds, more and more New Chemical Identities  
10 ("NCI") have been identified. There is thus an urgent need to establish a systematic method to study the mechanism of action of those NCIs in a high throughput fashion.

In one embodiment of the invention, in-cell macromolecular labeling is utilized to analyze the target of action of those candidate compounds with regard to different the biosynthetic pathways. A non-limiting example of one method is  
15 summarized briefly as follows: Radio-labeled building-blocks of different macromolecules are incubated with yeast and the incorporation of these radio-labeled building-blocks is measured either in the presence or in the absence of the compounds of interest. The compounds that inhibit a specific macromolecular synthetic pathway can be quickly identified and further characterized.

20 This method is especially powerful when applied to an *S. cerevisiae* metal ion regulated strain of the invention that can transiently reduce the expression of a target gene in the presence of metal ion. When the target gene is transiently knocked out, the synthetic pathway that involves that gene will be affected immediately and the incorporation of the building-blocks will be reduced. This effect can be used to  
25 characterize particular phenotypes associated with the target gene of interest. Analysis of antifungal compounds using the macormolecular assay described above and comparing the results to those obtained with the regulatable strains of the invention allows for the rapid determination of a phenotype for the candidate antifungal. Information about

expected phenotypes for antifungal compounds is thus further augmented when analyzing a compound that specifically inhibits the function of a given target gene.

One example of the methodologies used in these analyses of antifungal compounds is as follows. Similar methods may be employed when analyzing the regulated yeast strains of the invention.

#### Preparation

##### **Culture:**

Yeast is grown at 37°C overnight in a rotary bath in YNB medium with 0.05% glucose, 0.05% Difco Casamino acids, 0.1mg/L adenine and 0.14 mg/L N-acetylglucosamine. 5 ml of overnight culture is added to 25 ml of the same media until the O.D. reaches 0.5.

##### **Radiolabeling:**

N-Acetyl-D(1-3H)glucosamine	Amersham: TRK376, 9.9 Ci/mmol, 1 mCi/ml
D-(5-3H)Glucose	Amersham: TRK290, 17.4 Ci/mmol, 1 mCi/ml
L(4,5-3H)Leucine	Amersham: TRK510, 148Ci/mmol, 1mCi/ml
(2-3H)Adenine	Amersham: TRK311, 21 Ci/mmol, 1mCi/ml

20

Five different tubes containing the following supplements are setup:

1. DNA: 40 µl radiolabeled Adenine + 8 µl unlabeled Adenine.
2. Glucan: 40 µl radiolabeled glucose
3. Chitin: 40 µl radiolabeled N-acetylglucosamine + 4 µl unlabeled N-acetylglucosamine
4. Protein: 16 µl radiolabeled Leucine
5. RNA: 10 µl radiolabeled Adenine + 10 µl unlabeled Adenine.

25

**Reaction-stop:**

Setup 96-well plate for different stop-reaction:

1. Protein synthesis can be stopped in the plate with 75  $\mu$ l of 10% TCA in each well.
  - 5 2. RNA synthesis can be stopped in the plate with 75  $\mu$ l of 10% TCA in each well too but has to be in different plate with protein.
  3. DNA, Glucan and chitin synthesis can be stopped in the same plate with 75  $\mu$ l of 12% KOH.
  - 10 Holes should be drilled on each corner of the plates for better floating on water bath later on.
- Put the plate on ice to make reaction stop better.

**Compounds:**

- 15 For each macromolecular synthesis, three different concentrations (including 0  $\mu$ g/ml) of the testing compound are setup in three different tubes.

**Reaction:**

Each time point is be duplicated.

- 20 Four mls of the yeast culture are added to each different tube and timer is started to click. Take 75  $\mu$ l of each individual radiolabeling as background.

After incubation for 5 minutes, 1.2 ml of each reaction is added to the tubes containing testing compounds and 75  $\mu$ l of reaction is immediately taken to stop solution.

25

Repeat stop-reaction at 15, 20, 25, 35, 45, 55 minutes.

**Post-reaction:**



**Precipitation:**

Plates measuring DNA, glucan and chitin synthesis are heated for 90 minutes at 80°C. Then add 24 µl of 37% HCl and 19.3 µl of cold 50% TCA, incubated at 4°C for 30 minutes.

5

Plates measuring RNA synthesis are incubated at 4°C for 30 minutes.

Plates measuring protein synthesis are heated for 15 minutes at 80°C and then incubated at 4°C for 30 minutes.

10

**Filtration:**

Plates are filtered using GF-A plates usually machine will give better results. Wash twice with 200 µl 10% TCA and incubated at 65°C to let them dry.

15

**Count:**

Add 20 µl of scintillation fluid and counted

**References:**

For DNA, RNA and Protein synthesis:

20

Landini P, Corti E, Goldstein BP, Denaro M. Mechanism of action of purpuromycin.

*Biochem J.* 1992 Jun 15;284 ( Pt 3):935.

For Glucan synthesis:

25

Baguley BC, Rommele G, Gruner J, Wehrli W. Papulacandin B: an inhibitor of glucan synthesis in yeast spheroplasts. *Eur J Biochem.* 1979 Jul;97(2):345-51.

For RNA and protein:

Onishi JC, Milligan JA, Basilio A, Bergstrom J, Curotto J, Huang L,

Meinz M, Nallin-Omstead M, Pelaez F, Rew D, Salvatore, M, Thompson J, Vicente F, Kurtz MB. Antimicrobial activity of viridiofungins. *J Antibiot* (Tokyo). 1997 Apr;50(4):334-8.

5                   The following examples are intended as non-limiting illustrations of the present invention.

**Example 1: Construction of a Copper-Tolerant Yeast Strain Containing a Copper-Regulated ROX1 Gene**

10                   The following experiments were performed to: (i) examine the copper sensitivity of different yeast strains; and, (ii) to create a copper-dependent yeast strain containing a copper-regulatable ROX1 gene.

                  The copper sensitivity of yeast strain CTY145 was compared with that of ZMY60 by growing each strain in the presence of increasing amounts of added copper and measuring cell number at increasing times. After 16 hours of growth, the highest  
15                   concentration of copper at which the log-phase growth rate of ZMY60 was maintained unaffected was 250uM. (Figure 1). By contrast, the highest concentration of copper at which the growth rate of CTY145 was maintained was 1 mM. Thus, CTY145 is at least four-fold more tolerant to copper than ZMY60.

20                   A strain based on CTY145 that contains a copper inducible ROX1 was constructed as follows. Using a conventional lithium acetate/polyethylene glycol technique, CTY145 was transformed with approximately 0.1 ug of plasmid pZM195 (Figures 4A-D), which was linearized with the restriction enzyme AflII. The plasmid contains a metal responsive element comprising HIS3 promoter sequences fused to  
25                   ACE1.

Integration of the plasmid into the genome was monitored by the ability of the cells to grow on -URA plates (growth of the yeast strains in this medium requires the functional URA3 gene contained on the plasmid). After 48 hours at 30°C, the transformants were inoculated onto fresh -URA plates and regrown for an additional 48  
5 hours at 30°C. Well spaced individual colonies were picked and inoculated to 5 ml YPD media, grown overnight at 30°C, and 5 µl of the culture was inoculated into 5-FOA plates. In the presence of a functional URA3 gene, 5-FOA is converted to a toxin which kills cells. Therefore, the only cells that survive are those that have lost the URA3 gene by recombination.

10 Integration of ZM195 into the genome could occur in any of the following ways: (1) The original integration could occur non-specifically. In this case, the 5-FOA-induced deletion of URA3 would have no effect. (2) The original incorporation could occur specifically, and the subsequent 5-FOA induced deletion would result in a return to the original promoter sequence. (3) The original integration could occur specifically,  
15 and the subsequent 5-FOA induced deletion of URA3 could lead to the correct insertion of the copper-inducible promoter directly upstream of the ROX1 open-reading frame (ORF).

To detect the correct promoter insertion, three PCR primers were designed: ROX-A (5'-TCACACAAAAGAACGCAG-3') (SEQ ID NO:4), corresponding  
20 to a sequence from the region of the original promoter immediately 5' to the first ATG of the ORF; ROX-B (5'-GATGACAGCTGTGGTAGG-3') (SEQ ID NO:5), the reverse complement of a sequence in the ORF of ROX1 which is not present in ZM195; and ROX-C (5'-TCTTGCCATATGGATCTG-3') (SEQ ID NO:6), a sequence internal to the copper inducible promoter. For possibilities 1 and 2 above, PCR amplification of

genomic DNA with ROX-A and ROX-B would lead to a 601 base pair (bp) product, and PCR amplification with ROX-B and ROX-C would yield no product. For possibility 3, the correct insertion, PCR using ROX-A and ROX-B would yield a 2628 bp product, and PCR with ROX-B and ROX-C would yield a 785 bp product. PCR analysis identified  
5 a strain that had undergone the correct rearrangements. This strain was designated CUY101.

To bring the UBR1 gene under the control of the copper inducible promoter, HIS3-ACE1, the above-described procedure was repeated using CUY101 as the starting strain. The ZM197 plasmid (Figures 5A-D) that had been linearized by  
10 digestion with the restriction enzyme AatII was introduced into the cells. To identify cells in which the correct promoter insertion had occurred, three PCR test primers were designed: UBR-A (5'-ATCTTCGGACAAAGGCAG-3') (SEQ ID NO: 7); UBR-B (5'-GTGTAATTTTCGGGATCG-3') (SEQ ID NO:8) and ROX-C (5'-TCTTGCCATATGGATCTG-3') (SEQ ID NO:9). PCR analysis is used to identify one  
15 culture which has undergone the correct rearrangements. This strain is designated CUY103.

#### **Example 2: Construction of a Yeast Strain Containing A Deletion of SLF1**

In practicing the present invention, it is preferred that copper regulation  
20 of expression be maintained over a relatively long time period, i.e., for several days. The transient effect of copper in wildtype yeast is due at least in part to the fact that yeast cells are able to biomineralize copper. Thus, over time wild-type yeast cells deplete the medium of copper and the effect on expression is lost. If biomineralization activity is ablated, then the extracellular copper levels should remain nearly constant over time.

25 The only known gene in the yeast copper ion biomineralization pathway is the SLF1 gene. Inactivation of the SLF1 gene has been shown to result in cells which are slightly more sensitive to copper but are unable to efficiently deplete copper from the

media (Yu et al., *Mol. Cell Biol.* **16**:2464, 1996). Therefore, the SLF1 gene in the yeast strains described in Example 1 above was inactivated.

A construct was created for a two-step knockout of the SLF1 open reading frame. Primers SLF-E (5'-GCGCTGCAGGTCGACTTAGCAGGCAGTTTGAAC-3') (SEQ ID NO:10) and SLF-F (5'-GCGCTGCAGGCATGCACTCCTTTCCAATTGTGC-3') (SEQ ID NO:11) were used to amplify the 3'- untranslated region of SLF1 using genomic DNA as template. The Sall/SphI fragment of the PCR product was subcloned into Sall/SphI-digested pUC19 plasmid (Genbank accession no. M77789). This recombinant plasmid was designated pSLF3'. Similarly, primers SLF-G (5'-GCGAGCTCGGTACCCCATACCCCTAACTCTAG-3')(SEQ ID NO: 12) and SLF-H (5'-GCGGATCCCGGGGCTCTCTCGTTTATTTAACG-3')(SEQ ID NO:13) were used to amplify the 5'- untranslated region of SLF1, and the SacI/BamHI or KpnI/BamHI fragment was cloned into SacI/BamHI or KpnI/BamHI digested pSLF3' to produce pSLF3'5'. The 5.5 kb BamHI/XbaI insert of pDJ20, which contains the yeast URA3 gene and bacterial kanamycin resistance gene flanked by a direct repeat of the *Salmonella* HisG sequence, was subcloned into XbaI/BamHI digested pSLF3'5' to create pSLFKO. Plasmid pDJ20 is derived from the plasmid pSP72 (Promega, Madison, WI) into the BamHI site of which has been cloned the approximately 5.5. kb insert consisting of the following elements:

20 | hisG | URA3 | kanamycin resistance | hisG |

The hisG elements are present as a tandem repeat. Plasmids containing this element can be transformed into bacteria for amplification; selection with kanamycin helps to avoid unwanted recombination between the two hisG regions in bacteria which would result in the loss of the *S. cerevisiae* URA3 gene. The hisG, URA3 and kanamycin genes are

well-known in the art and can be assembled in this order by conventional techniques in molecular biology, and do not need to be obtained from plasmid pDJ20.

This plasmid (pSLFKO) was digested with SphI and EcoRI and transformed into strains ZMY60, CTY145, CUY101, and CUY103 using a conventional  
5 lithium acetate/polyethylene glycol technique, as described above in the Methods section. Integration of the plasmid into the genome of each yeast strain was monitored by the ability of the strain to grow on (-)URA plates. After 48 hours at 30°C, the transformants were inoculated onto fresh (-)URA plates and regrown for an additional 48 hours at 30°C. Well spaced individual colonies were inoculated into 5 ml YPD media, grown overnight  
10 at 30 °C, and 5 µl of the culture was inoculated onto 5-FOA plates.

In the presence of a functional URA3 gene, 5-FOA is converted to a toxin which kills cells. Therefore, the only cells that survive are those which have lost the URA3 gene by recombination. Either of the following could occur: (1) Non-specific integration of the linear DNA containing the 5'-HisG-URA3-kanR-HisG-3'NTS fragment  
15 could occur, followed by deletion of the region between the HisG repeats; this would result in a 5'NTS-HisG-3'NTS integration at some random spot. (2) Alternatively, specific integration of the linear DNA containing the 5'NTS-HisG-URA3-kanR-HisG-3'NTS sequence could occur, followed by deletion, which would result in a deletion/insertion in which the entire ORF of SLF1 has been deleted and a single copy  
20 of the HisG element has been left in its place.

To confirm that the correct genetic alteration occurred, PCR was performed using the following sets of primers: (i) HISGCH (5'-GATTTGGTCTCTACCGGC-3') (SEQ ID NO: 14) and SLF-D (5'-GACAGTATCGTAATTACG-3') (SEQ ID NO: 15); and (ii) a primer comprising the  
25 reverse complement of primer HISGCH and SLF-D as above. Alternatively, PCR with SLF-A (5'-CTAACTCTAGCTGCATTG-3') (SEQ ID NO: 24) (or SLF-G) and SLF-D (or SLF-F) could be used to produce a diagnostic shift in product length after PCR. The SLF1 deleted version of CTY145 is designated CUY104; the SLF1 deleted version of

CUY101 is designated CUY105; the SLF1 deleted version of CUY103 is designated CUY106; and, the SLF1 deleted version of ZMY60 is designated CUY107.

Cultures of yeast strains CTY145, CUY101, CUY103, CUY104, CUY105, and CUY106 were cultured in 5 ml of complete synthetic media (CSM) supplemented with 500 mM  $\text{CuSO}_4$  at 30 °C in a rollerdrum apparatus at a speed of approximately 60 revolutions per minute. At 24 and 48 hours, the cultures were pelleted and resuspended in fresh CSM media containing 500 mM  $\text{CuSO}_4$ . After 96 hours of incubation, cells were collected onto filter paper and the supernatant was removed by suction through the filter paper. Biom mineralization is inferred by the presence of a darkened cell pellet, indicating the biom mineralization of the soluble copper to copper sulfide ( $\text{CuS}$ ) which has been shown to be deposited on the cell surface. Strains CTY145, CUY101, and CUY103 (A, B, and C, respectively in Figure 9) contain the wild-type SLF1 gene, as demonstrated by their dark color. Strains CUY104, CUY105, and CUY106, in which the SLF1 gene has been deleted, show considerably lighter coloration after collection on filter paper, indicating an ablation of copper biom mineralization activity.

Single colonies of each yeast strain described above were picked from YPD plates and grown overnight in YPD media at 30 °C with shaking. Cultures were diluted to an absorbance at 600 nm of 0.02 in CSM media containing various concentrations of  $\text{CuSO}_4$ . Cultures were grown for 24 hours at 30 °C in a rollerdrum apparatus at approximately 60 revolutions per minute. The absorbance of each culture at 600 nm, which is a measure of cell density (i.e., the number of cells in a culture) was measured. The results of the cell density assays are shown in Figure 10, and is expressed as a percentage of the cell density achieved in cells with no added copper sulfate.

**Example 3: Method for Stable Replacement of the Promoter Element of Any Gene of Interest with a Copper-Inducible Promoter**

The following procedures were performed to stably replace the promoter  
5 element of any yeast gene of interest. The strategy is designed to avoid: (1) the use of  
URA3 as a selectable marker, which precludes its use in future selection procedures; (2)  
the requirement for a naturally occurring unique restriction site in the coding sequence  
of the subject gene; (3) the need for multiple subclonings; and (4) the need for constant  
maintenance of URA3 selection in order to prevent loss of the inserted sequence, which  
10 would result in restoration of the original promoter elements.

A single or double-PCR strategy was devised. Instead of a single  
crossover event, the method requires that a double crossover occur in order to achieve  
integration into the yeast genome. Although a double crossover event is less likely to  
occur, once it has occurred the resulting transformed haploid yeast strain does not have  
15 to be maintained under selection. HIS3 is used as a marker to avoid using URA3  
unnecessarily; in addition, the HIS3 gene is relatively short and therefore comparatively  
easy to amplify.

A plasmid designated pCU3 was constructed which contains a functional  
HIS3 gene (including upstream sequences) in inverted orientation to, and upstream from,  
20 the ANB1 promoter. The ANB1 promoter was fused upstream (i.e., 5' to) sequences  
encoding ubiquitin tag elements. For this purpose, the BamHI/PstI fragment of  
pUC8-Sc2676 was subcloned into pUC19. Then, the EcoRI-KpnI fragment of ZM168,  
which contains the ANB1 promoter and the ubiquitin tag regions, was subcloned into the  
plasmid.



Primers were designed for use in either a one-step or two-step PCR strategy. (Figures 2A-C).

*One-Step PCR Strategy:* 100-mer oligonucleotides were synthesized. Primer 1 contains 80 bp of target sequence from the gene of interest, which is obtained  
5 from knowledge of the DNA sequence immediately 5' to the protein-coding sequence of the gene, together with 20 bp of plasmid sequence. A second set of primers comprising sequences from the non-coding strand is synthesized. These oligonucleotides, which are designated either Primer 2b (5'-CCAGACTACGCTTCGATATCG-3') (SEQ ID NO: 16) ("Tag") or Primer 2a (5'-CACAATAAAACATCGATATT-3') (SEQ ID NO: 17) ("NO  
10 TAG"), are then fused to 18-20 bp of the protein-coding sequence of a gene of interest, beginning with the initiator ATG codon. In this case, "Tag" refers to the presence or absence of the ubiquitin tag.

Primer pairs 1 and 2a or 1 and 2b are used to amplify a DNA fragment from pCU3, producing a fragment consisting of genomic 5'NTS (non-translated  
15 sequence) followed by HIS3 in an inverted orientation, ANB1 promoter, and either a fragment of the ORF or a tag sequence fused in frame to a fragment of the ORF. Transformation of haploid yeast strains with these sequences, followed by double crossover, leads to integration into the genome. This results in insertion of HIS3, the ANB1 promoter and (in some cases) the tag sequence 5' to the gene of interest.

20 Using this approach, no DNA sequence is lost and no sequence is duplicated, thereby considerably lessening the likelihood that the inserted sequence will be spontaneously deleted. After selection with HIS3, the presence and orientation of the insert is confirmed using PCR. Because the integration requires a double crossover, selection by HIS selection should not be required to maintain the genotype.

25 *Two-Step PCR Strategy:* For the two step strategy, Primer 2a or 2b is fused to 18-20 bp of the ORF of a gene of interest, beginning with the initial ATG of the

ORF. Either primer, and a second primer comprising 18-20 nucleotides that is the reverse complement of a sequence 400-1000 bp downstream, are used to amplify a 400-1000 bp fragment that is the reverse complement of the ORF and has a 3' tag complementary to the sequence in pCU3 such that the sequence is fused in frame to the tags or is fused in frame in place of the tags.

The fragment corresponding to the opposite end is produced by fusing a primer designated "Universal HIS3-2STEP" (5'-CAGGCATGCAAGCTTGGCGT-3') (SEQ ID NO:18) to an 18- to 20-mer representing the reverse complement immediately 5' of the starting ATG of the ORF. This fragment is used in conjunction with a primer identical to 18-20 nucleotides comprising a sequence 400-1000 bp 5' to the starting ATG to amplify a fragment whose 3' end is complementary to the 3' end of the HIS3 gene in pCU3.

The two fragments are then used to amplify pCU3, producing a fragment comprising a length of genomic 5'NTS followed by HIS3 in inverted orientation, ANB1 promoter and either a length of the ORF or a tag sequence fused in frame to a length of the ORF. Transformation with this sequence and double crossover leads to integration into the genome, which results in the insertion of HIS3, the ANB1 promoter and (in some cases) the tag sequence. Using this approach, no DNA sequence is lost and no sequence is duplicated, thereby greatly lessening the likelihood of spontaneous deletion. After selection with HIS3, the presence and orientation of the insert is confirmed with PCR. Because the integration requires a double crossover, HIS selection should not be required to maintain the genotype.

**Example 4: Alternative Method for Stable Replacement of the Promoter Element of Any Gene of Interest with a Copper-Inducible Promoter**

PCR primers are designed to amplify sequences of the target gene to result in a “shuffled gene” arrangement (Figure 6) in the vector pCU19Srf (Figure 8). The PCR primers that can be used will vary from 18 to 36 nucleotides in length, and will preferentially have a GC content of at least 50 percent. When primers, which are 18 to 36 nucleotides in length have a low GC content, the primers can be made 3 to 6 nucleotides longer than those with at least a 50% GC content.

PCR primers are dissolved in sterile water at a concentration of 1 mg/ml. Genomic DNA from *S. cerevisiae* is diluted to various concentrations in sterile water. Taq DNA polymerase (Promega Biotech, Madison, WI) is typically used for the primary PCR reaction, but other thermostable polymerases, such as Vent polymerase (New England Biolabs, Beverly, MA) or Pfu polymerase (Stratagene, Carlsbad, CA) can also be used.

The shuffled gene of interest is generated by performing two primary PCR reactions. In one, a portion of the target gene which starts about 400 base pairs upstream of the ATG start codon and ends just upstream of the ATG start codon is amplified. In the second primary PCR reaction, a portion of the target gene that starts at or that is just upstream of the ATG start codon and that ends about 400 base pairs downstream of the ATG start codon is amplified. A typical primary PCR reaction will include 10 µl of 10X Taq buffer, 10 µl of 25 mM deoxynucleoside triphosphates, 10 µl of 25 mM MgCl<sub>2</sub>, 1 µl of *S. cerevisiae* genomic DNA, and 1 µl of primer pairs (Primers 1 and 2, or primers 3 and 4) at 100 µg/ml, and 66 µl of sterile water.

Typically, primer 1 will consist (from the 5' to the 3' end) of a seven nucleotide sequence, then a NotI restriction endonuclease cleavage site (or a restriction site susceptible to cleavage by another rare-cutting enzyme), followed by 10 or 11 nucleotides which are identical to the top strand of the gene of interest about 400 base pairs upstream of the ATG start codon of the target gene. The seven nucleotides at the 5' end of primer 1 are complementary to the 7 nucleotides immediately 3' of the NotI site in primer 4.

Primer 2 will have the sequence of the bottom strand of the gene of interest, just upstream of the ATG start codon, and will comprise about 18 to about 21 nucleotides.

Primer 3 will have the sequence of the top strand of the target gene either at or very close to the ATG start codon.

Primer 4 will consist of a seven nucleotide sequence at the 5' end, followed by a NotI restriction endonuclease cleavage site (or a restriction site susceptible to cleavage by another rare-cutting enzyme), followed by 10 or 11 nucleotides which are identical to the bottom strand of the gene of interest about 400 base pairs downstream of the ATG start codon. The seven nucleotides at the 5' end of primer 4 are complementary to the 7 nucleotides immediately 3' of the NotI site in primer 1.

Typically, the reaction is initiated by heating the reaction mixtures to 94 °C for 3 to 5 minutes, followed by the addition of 5 units of Taq DNA polymerase. The reaction is then thermocycled reaction mixture through 1 minute at 94 °C, 1 minute at 50 °C, and 2 minutes at 72 °C for 30 cycles, after which the temperature is reduced to 4 °C. The PCR products are run on an agarose gel to determine the conditions that produced a fragment of the appropriate size, typically about 400 base pairs. Modifications of

various parameters of the method in order to optimize reaction conditions, such as altering annealing temperatures, salt concentrations, and the like are within the skill of the ordinarily skilled worker.

The secondary PCR reaction uses the primary PCR reaction products as  
5 DNA templates. The one end of each of the two primary PCR products are homologous, and when melted, will anneal to each other over a stretch of about 21 base pairs. The primers used for the secondary PCR reaction are primers 2 and 3 used in the primary PCR reactions. Use of these primers will anneal to the ends of the annealed template DNA and allow the PCR reaction to produce a shuffled gene reaction product.

10 A typical secondary PCR reaction will include 10  $\mu$ l of 10X Pfu buffer, 10  $\mu$ l of 25 mM deoxynucleoside triphosphates, 1  $\mu$ l of primer pairs (i.e., primers 2 and 3 from the primary PCR reaction) at a concentration of 100  $\mu$ g/ml, 77  $\mu$ l of sterile water, various dilutions of the primary PCR products, and 1  $\mu$ l of Pfu polymerase, comprising 2.5 activity units. The PCR conditions are identical to those to be used in the primary  
15 reactions, except that the initial heating of the tubes to 94 °C for 3 to 5 minutes is omitted.

The secondary PCR product is electrophoresed through an agarose gel, according to methods well-known in the art, and the appropriate band (of about 800 to 900 base pairs) is cut out. The DNA is then extracted from the gel using, e.g., the Gene  
20 Clean kit (Bio101, Vista, CA). The extracted, purified DNA is then used for ligation into the vector pCu19Srf (Figures 8).

To perform the ligation, the PCR-Script kit from Stratagene (La Jolla, CA) is used. pCu19Srf is cut with SrfI restriction endonuclease. The ligation mix contains 100 ng of SrfI cut vector DNA, 1  $\mu$ l of 10X PCR Script buffer, 0.5  $\mu$ l ATP, 4 to 6  $\mu$ l of

insert DNA, containing from 100 to 500 ng, 1  $\mu$ l of T4 DNA ligase, and 1  $\mu$ l of SrfI. All reagents are provided in the PCR Script kit except for the DNA. However, such reagents are well-known and commercially available from other sources. Three  $\mu$ ls of the ligation reaction is transformed into competent DH5 $\alpha$  cells, which can be obtained from  
5 Gibco/BRL (Rockville, MD), and the cells are plated on LB medium with 100  $\mu$ g/ml ampicillin. The plates are incubated at 37 °C for 16 to 18 hours. Single colonies are chosen for restriction enzyme digestion and analysis of the resulting fragments to identify a clone which contains the insert in the proper orientation. A colony that is identified as containing a plasmid with the insert in the proper orientation is selected and is amplified  
10 by culturing. The insert-containing plasmid is purified from the bacterial host using the DNA isolation procedure of the Qiagen DNA preparation kit (Qiagen, Hilden, Germany) or other well-known methods for plasmid purification. The purified DNA is digested with NotI restriction endonuclease (or another rare-cutting endonuclease whose restriction site has been engineered into the shuffled gene). The endonuclease is  
15 inactivated by heating for 20 minutes at 65 °C. The purified, cut DNA is then used to transform *S. cerevisiae*.

Strain CUY106 (Ace-ROX1, AceUBR1, deltaSLF1, his3delta200, leu2-3,112, ura3-52) is transformed by standard methods with the NotI digested plasmid DNA (Figure 7). Cells are plated on CSM agar lacking histidine and are incubated at 30 °C for  
20 40 to 48 hours. Single colonies are selected and restreaked for single colonies on the same media. A culture from a single colony is grown in YPD and a genomic DNA is isolated for evaluation by PCR reaction to verify the construction of the strain.

PCR primers for genomic verification are designed so that one primer (Primer 5') at one end of the gene is 5 prime to Primer 1 (above) used to generate the

shuffled gene on the plasmid and on the same strand as Primer 1. Another primer (Primer 3') is designed which is 3 prime to Primer 4 (above) and on the same strand as Primer 4. PCR conditions are as those described for Primary PCR (above). Another primer, specific for the plasmid sequence, 5'-ACCCTGGCGCCCAATACG-3' (SEQ ID NO:23), is used in conjunction with Primer 3' to amplify DNA from the mutant strains, i.e., those that contain the shuffled genes. The product of this PCR reaction is typically 600 to 700 base pairs in length.

Wild type genomic DNA and Primer 5' and Primer 3' are used to amplify a 1 to 1.5 kb PCR product. This primer pair should not yield a PCR product from the mutant genomic DNA using these PCR conditions because the product would be too big to amplify (>7 kb).

**Example 5: Assay for Reversion Frequency in Yeast Strains Engineered with Copper Repressible Genes**

The following assay was performed to assess the frequency with which a culture maintained under non-selective conditions will revert to a phenotype of non-sensitivity to exogenously added metal ions. Cultures of strains produced by two different methods in different strain backgrounds were grown in the absence of selection, then assayed to determine what percentage of the cells in the culture were no longer sensitive to the addition of copper ions.

Two independent isolates of ZMY71(ZM71 #1 and ZM71 #2) were used in this assay. ZM71 is derived from ZMY60, and its construction is described in Moqtaderi, Z. et al., *Nature* 383:188-191 (1996). The SUA7 gene was operably linked to an ANB1 promoter by a single cross-over strategy in a strain in which ROX1 and UBR1 are activated by the addition of copper to the culture medium. The recombinant strains are

maintained by selection on media lacking uracil (-URA). It is known that in the absence of selection, spontaneous recombination results in a strain in which the URA3 gene is lost (reverting to a ura3 phenotype) and regulation of the SUA7 gene by the ANB1 promoter is lost, while wild-type regulation of SUA7 is restored.

5                   Two independent yeast strain isolates (19SG1 and 19SG2) were also used. In these yeast strains the SUA7 gene in strain CUY106 was operably linked to an ANB1 promoter by the double cross-over strategy as detailed in Example 4. The recombination results in a strain that can be selected for on media lacking histidine (-HIS). Because of the method used to engineer this strain results in an insertion which does not contain any  
10 tandem repeats of sequence, it should be less likely that in the absence of selection on -HIS media the strain would revert to a his3 phenotype or regain wild-type regulation of the SUA7 gene.

                  All strains were streaked from glycerol stocks to the appropriate selective media (uracil free media for ZM71 and histidine free media for the 19SG strains) and  
15 were grown for 72 hours at 30°C. Single colonies were picked and inoculated into 2 ml of selective media and were cultured overnight in a rollerdrum at 30°C. The yeast cultures were microcentrifuged for approximately 5 seconds and the pellets were resuspended in two ml of YPD media (non-selective). The cultures were then grown 24 hours at 30°C in a rollerdrum.

20                   Dilutions of each culture were plated to YPD and CSM plus 1 mM cupric sulfate plates. Plates were incubated for 72 hours at 30°C and the colonies were counted.

YPD plate colony numbers reflect the total cells in the culture, while colonies on the CSM plus 1 mM cupric sulfate plates indicate revertants, i.e., cells which have become



insensitive to the copper ion stimulus. Revertants are expressed in the table below as a percentage of total cells observed.

	<u>STRAIN</u>	<u>REVERTANTS</u>
5	ZM71 #1	0.012%
	ZM71 #2	0.22%
	19SG1	0.00024%
	19SG2	0.00042%

10                    Not all reversions are due to genetic changes at the SUA7 locus. It is also possible that the copper stimulation of UBR1 or ROX1 gene expression can be ablated. However, since the control of these genes is identical in all the strains, any ablation of copper stimulation of these genes appear as background which all strains will share. The change in the reversion frequency at 24 hours in the 19SG strains engineered according  
15 the methods of Example 4 demonstrates the improvements that can be achieved by altering gene expression in yeast according the methods disclosed herein.

**Example 6: Construction of Yeast Strains Containing CYC8-LexA Repressor**  
**Under Copper Control**

20                    The following procedures are performed to produce a yeast strain that expresses a heterologous repressor under copper control. Such strains avoid the potential problems of ROX1-based repressor strains, which include the pleiotropic effects and toxicities of ROX1 and metal ions. In a LexA-based repressor system, the addition of metal ions represses only recombinant genes whose promoters have been engineered to  
25 contain the bacterial-derived recognition sequence for LexA (LexA operator). A CYC8-LexA fusion has been shown to repress the transcription of a yeast gene when the

LexA operator sequence is placed adjacent to the promoter of the yeast gene (Keleher et al., *Cell* **68**:709, 1992).

This system comprises two components: (i) a yeast strain which, in the presence of copper, expresses a CYC8-LexA fusion protein or a fusion protein between  
5 LexA and a fragment of ROX1 that lacks DNA-binding activity and (ii) a DNA fragment which renders any desired target gene repressible by LexA when introduced upstream of the start of the open reading frame. Notably, this can be achieved even if only a limited amount of sequence information is available.

A repressor fusion protein is constructed so that the DNA-binding domain  
10 of LexA (amino acids 1-87) is fused to the N-terminus of the entire CYC8 protein (amino acids 1-966) as well as 23 amino acids derived from the 5' untranslated region of CYC8. This hybrid protein is expressed from a conditionally "inert" locus, such as TRP1. Alternatively, a fusion protein is constructed so that the DNA-binding domain of LexA is fused to a ROX1 protein which has been mutated so that it no longer binds to ROX1  
15 recognition sequences, such as those present in the yeast ANB1 promoter.

In order to render a particular gene repressible by the hybrid repressor proteins described above, a generic repressible promoter cassette is designed that can be inserted upstream of any gene. The promoter cassette consists of one to several copies of the LexA operator placed upstream of a UAS-containing yeast promoter, such as, e.g.,  
20 CYC1, adjacent to a gene such as HIS3 which can be used for positive selection. The insertion cassette can be produced by a single- or double-round PCR strategy by analogy to the method shown in Figures 2A-C. In this case, the point of insertion is upstream of the promoter region and not at the translational start site (Figure 3).

The repressible promoter is modified so that it will integrate upstream of a given yeast target gene as follows. Four PCR primers are designed based on limited sequence data flanking the 5' end of the gene of interest as shown in Figures 2A-C.

5 (i) Primer A is located 100-200 base pairs upstream of the beginning of the open reading frame of the gene, oriented toward the gene.

(ii) Primer B is located 100-200 within the 5' end of the open reading frame, oriented towards the 5' start of the gene.

(iii) Primers C and D contain both sequences specific to each gene as well  
10 as sequences homologous to the generic repressible promoter cassette. Primer C contains at its 5'-most end sequences corresponding to the 3' end of the promoter cassette, in this case the 3' end of the CYC1 promoter. The 3' half of primer C contains sequences corresponding to the 5' end of the open reading frame of interest. The 3' end of Primer D consists of the sequence complementary to the sequence just upstream of the gene of  
15 interest. The 5' half of Primer D consists of sequences complementary to the left-most end of the promoter cassette, in this case the 3' end of the HIS3 gene.

Typical sequences for primers C and D are:

Primer C: 5

ACAAATACACACACTAAATTAATAATGNNNNNNN-3' (SEQ ID NO:19)

20 Primer D: 5'-end of HIS3- NNNNNNNNNNNNNNNNNNNNNNNN-3'

Two sets of PCR reactions are performed. In the first set, a fragment of DNA containing the gene of interest and 5' flanking region is used as a template and amplification is performed using Primers A and D or Primers B and C. The resulting fragments are then included in a second round of PCR containing the promoter cassette,  
25 both initial PCR products, and Primers A and B. This results in a larger fragment

containing the promoter cassette flanked by pieces of DNA that will target the DNA just upstream of the gene of interest (Figure 3).

Following transformation into yeast and selection for integration, stable integrants produced by sequence-specific recombination into the site of interest can be positively identified by PCR or Southern blot analyses. This strategy produces a conditional locus that is repressible by the LexA fusion proteins described above.

**Example 7: Demonstration of Cidal and Static Effects of Copper Ion-induced Repression of Gene Expression**

Three different yeast cell strains were constructed based on the CUY106 strain. The yeast CDC15, SUA7, and ERG11 genes were rendered repressible by copper-ion addition to the growth medium as described above.

The viability of each strain was evaluated at several time points over an 8 hour period after the addition of 1 mM copper sulfate to the growth medium by diluting the cells and plating them on YPD medium, without copper sulfate. Yeast colonies were counted after 48 hours of incubation at 30 °C in order to determine the colony forming units (CFU) per ml of original copper sulfate-containing culture medium at the time the yeast cells were harvested and diluted. The CFU/ml value was divided by the measured absorbance of the original copper sulfate-containing growth medium at 600 nm ( $A_{600}$ ) at the time the aliquots were taken for dilution and plating onto YPD medium, yielding a viability index of  $CFU/A_{600}$ . The limit of detection for  $CFU/A_{600}$  is approximately  $1 \times 10^5$ . Figure 11 shows the results of the assay, demonstrating that repression of the expression of some genes, such as SUA7 and ERG11, is cidal, i.e., kills, the yeast cells, while repression of expression of other genes, such as the CDC15 gene, only has a static effect on the yeast cells, arresting their growth but not killing them.

**Example 8: The Copper Regulated Strains Are Complemented**

Wildtype and mutant forms of BOS1 were created using PCR. The wildtype Bosl gene was amplified from *Saccharomyces* genomic DNA in a one-step reaction using the following oligos: ScBos1-1 (5'-GCGGCTCGAGGGGTTTCTCTCAACATTG-3') [SEQ ID NO: 25] and ScBos1-2 (5'-GATCGCGGCCGCGTAAGGCTTATTGCTGCG 3') [SEQ ID NO: 26]. The region cloned included the wildtype BOS1 promoter and contained XhoI and NotI restriction sites at the 5' and 3' end respectively to facilitate cloning. The mutant allele, bosl-200, is missing codons 9-130 and was created by PCR in two steps using four oligos. In the first round of amplification, the 5' region of the gene was amplified using oligos ScBos1-1 and ScBos1deIr (5'-CCACCAACGTTCTCACAGCATGGTTGTAAAGAGC-3') [SEQ ID NO: 27] and the 3' portion of the gene was amplified using oligos ScBosldelf (5'-AGGAACGTTGGTGGTGCG-3') [SEQ ID NO: 28] and ScBos1-2. In the second round, the two products of the first round of amplification were used as template and were amplified using primers ScBos11 and ScBos1 -2. The genes were cloned into the yeast expression plasmid pRS315 (Sikorski, R.S. and Hieter, P., *Genetics* 122 19-27, 1989) containing the LEU2 selectable marker and a CEN sequence that functions to limit the plasmid copy number to one or two copies per cell. The BOS1 copper regulated strain, Y360, was transformed with pRS315, pRS315 expressing wildtype BOS1p, and pRS315 expressing the bosl-200 mutant protein.

The three strains, Y360, Y360 expressing wildtype BOS1 and Y360 expressing the bosl-200 mutant protein were streaked for single colonies on CSM plates and CSM plates containing 1mM CUS04. The plates were incubated at 30°C for 3 days

to allow colony formation. The results of the complementation of growth experiment are shown in Figures 14a-c.

In the absence of copper, levels of BOS1p were sufficient in the Y360 copper regulated strain and cells grew normally. In the presence of copper, BOS1 protein levels dropped and the cells were unable to survive. Thus, the Y360 strain containing the pRS315 vector alone died in the presence of copper. The Y360 strain expressing wildtype BOS1 was capable of growth on copper. The Y360 expressing the mutant bosl-200 protein was also capable of growth on copper. These result suggest that the bosl-200 mutation is not deleterious under these conditions. These generally applicable analyses can be used to test a region or regions of a given target protein for its ability to complement. The same methods are used to test a non-homologous cDNA for its ability to complement a Cu-regulated mtuant target gene by inserting the nonhomologous gene into a plasmid and carrying out the transformation of a given strain of the invention according to the methods described herein.

#### **Example 9: Phenocopy control for compound treated cells**

Two different yeast cell strains were constructed based on the CUY106 strain. The yeast TAF145 (Y002) and RPC34 (Y038) genes were rendered repressible by copper-ion addition to the growth medium as described above. The parent CUY106 strain and the TAF145 strain were used as controls. RPC34 is an essential gene encoding the C34 subunit of RNA polymerase III. RNA polymerase III is a multisubunit enzyme responsible for the synthesis of tRNAs, 5sRNAs and some other small RNAs. Rpc34 temperature sensitive and cold-sensitive mutants have been shown to have defects in tRNA synthesis. (Stettler, S., *et al.*, *J. Biol. Chem.* 267 21390-21395, 1992; and Brun, I., *et al.* *EMBO J.* 16 5730-5741 1997.) TAF145 is a component of general transcription factor IID (TFIID) and is required for synthesis of some mRNAs but is not required for tRNA synthesis. All of the strains used in this experiments were assayed for the production of tRNA upon addition of copper to the media by monitoring the generation of tRNA precursor.

## Strains and Procedures:

Yeast Strains

CUY106 (Wild Type): Ace-ROX1, Ace-UBR1, Dslf1, his3D200, leu2-3,112, ura3-52

- 5 Y038 (RPC34): Ace-ROX1, Ace-UBR1, Dslf1, his3D200, leu2-3,112, ura3-52, ANB1p-Ub- Rpc34::HIS3

Y002 (TAF1145): Ace-ROX1, Ace-UBR1, Dslf1, his3D200, leu2-3,112, ura3-52, ANB1p-Ub-Taf1145::HIS3

10

Growing Yeast Strains

1. Grow yeast strains overnight in Yeast Extract Peptone and Dextrose Medium "YPD" at 30°C.
2. Dilute cultures in the morning to  $OD_{600}=0.1$  and let them grow for 1hr at 30°C.
- 15 3. Measure  $OD_{600}$  (equal to about 0.2) and save aliquots of each culture for "0hr" point. Spin each aliquot for 5min at 5,000rpm at 4°C, discard supernatant. Resuspend pellet in 1ml ice-cold water. Transfer to a clean 1.5ml microcentrifuge tube. Microcentrifuge 10sec at 4°C, and remove supernatant. Freeze the pellet by placing tube on dry ice, and then transfer it to -80°C for storage.
- 20 4. Split cultures in two halves and induce one half with 0.75mM CuSO<sub>4</sub>, continue growing both halves at 30°C.
5. Collect culture aliquots at 30min, 1hr, 2hrs, and 4hrs (or 3.5hrs) after induction with CuSO<sub>4</sub> (or a compound). Measure  $OD_{600}$  at each point (for growth curves). Wash cells in 1ml ice-cold water and freeze the pellet by placing tube on dry ice. Store at -  
25 70°C.

Preparation of Yeast RNA with Hot Acidic Phenol

1. Thaw pellet on ice. Resuspend pellet in 400µl TES solution (10mM tris-Cl, pH 7.5; 10mM EDTA; 0.5% SDS). Add 400µl acid phenol (Gibco BRL) and vortex vigorously 10min. Incubate 30 to 60 min at 65°C with occasional, brief vortexing.
- 5 2. Place on ice for 5 min. Microcentrifuge 5 min at top speed, 4°C.
3. Transfer aqueous (top) phase to a clean 1.5-ml microcentrifuge tube, add 400µl acid phenol, and vortex vigorously. Repeat step 2.
4. Transfer aqueous phase to a new tube and add 400µl of chloroform. Vortex vigorously and microcentrifuge 5min at top speed, 4°C.
- 10 5. Transfer aqueous phase to a new tube; add 40µl of 3M sodium acetate, pH 5.3, and 1ml of ice-cold 100% ethanol and precipitate. Microcentrifuge 5min at top speed, 4°C. Wash RNA pellet by vortexing briefly in 1ml ice-cold 70% ethanol. Microcentrifuge as before to pellet RNA.
6. Resuspend pellet in 50µl H<sub>2</sub>O (heat if needed for resuspending at 65°C).
- 15 Determine the concentration spectrophotometrically by measuring OD<sub>260</sub> and OD<sub>280</sub>. Store at -20°C.

$$(\text{RNA concentration} = \text{OD}_{260} / 0.0025 \mu\text{g/ml})$$

7. Equalize RNA concentration in all samples to 2µg/µl:
8. Run 2µl of each RNA sample on 0.7% agarose gel to visualize equal RNA
- 20 concentrations.

S1 Nuclease Assay

1. tRNA Oligonucleotide Probe Labeling

Mix 2µl H<sub>2</sub>O with 1µl of unlabeled 5µM oligo tRNA<sup>w</sup>



(5'-GGAATTTCCAAGATTTAATTGGAGTCGAAAGCTCGCCTTA-3' [SEQ ID NO:29]. This sequence is complementary to the 5' intron-exon junction of short-lived precursor tRNA (Cormack and Struhl, Cell 69, 685-696,1992).and contains six nucleotides at the 3' end which are noncomplementary to RNA in order to carry out the S1 nuclease assay.

Incubate at 70°C for 5min, cool on ice, spin to collect sample on the bottom.

Add to the tube:

2.5µl gamma-<sup>32</sup>P-ATP (>5000 Ci/mmol, 10 µCi/µl)

10µl 10x protein Kinase "PNK" buffer (NEB)

10 1µl PNK (NEB)

Incubate at 37°C for 45min.

Incubate at 70°C for 15min. Store on ice or at -200°C long term.

## 2. Hybridization

Use 40µg of RNA per reaction

15 RNA

10µl 5x hybridization buffer (1.5 M NaCl; 5mM EDTA; 190 mM HEPES, pH 7.0)

1µl 5% Triton X-100

1µl labeled probe

20 H<sub>2</sub>O to 50µl total volume

Incubate 15min at 75°C, and then 4 hours or overnight at 55°C

## 3. S1 Nuclease Digestion

After hybridization is complete, to 50µl hybridization mix add:

50µl 10x S1 buffer (3M NaCl; 20mM ZnOAc; 600mM NaOAc, pH 4.5)

2µl 5% Triton X-100

400µl H<sub>2</sub>O

150units S1 nuclease (0.375µl of 400U/µl S1 from Boehringer Mannheim)

Incubate at 37°C for 30min

- 5 Add 5µl of 10mg/ml in H<sub>2</sub>O carrier bakers yeast tRNA and 5µl of 0.5M EDTA, pH8.0, and precipitate with 1ml of 100% ethanol for 30min at -70°C. Spin 20min in a microfuge; remove supernatant and dry pellet on benchtop for 10-15min. Add 12µl sequencing loading buffer, heat 5min at 100°C and put on ice for 5min. Load 4µl on 10% TBE-Urea gel (BIORAD). Run at 200V for 20 minutes.
- 10 Dry gel in a vacuum drier for 2 hours at 80°C. Reveal results by autoradiography.

- Figures 15 demonstrates that, upon addition of CuSO<sub>4</sub>, RNA synthesis drops in the Cu-regulatable RPC34 strain (YO38). tRNA synthesis was not decreased upon addition of Cu in the control CUY106 strain or the TAF145 regulatable strain (Y002). This result is consistent with published reports on the role of RPC34 in tRNA synthesis. (Brun et al., *EMBO J*, **16**:5730, 1997) and demonstrates that the regulatable strains of the invention can be utilized to provide information regarding the phenotypes expected when antifungal compounds are isolated.
- 15

20 **Example 10: Drug Sensitivity Profiling**

- In *S. cerevisiae*, the essential gene *ALG7* encodes the enzyme Dolichol-P-dependent N-acetylglucosamine-1-P transferase (GPT). The GPT enzyme initiates the first step in the assembly of dolichol-linked oligosaccharide N-glycosylation. Kukuruzinska *et al.*, *Biochim Biophys Acta* **1247**(1) 51-59 (1995)
- 25 demonstrated that downregulation of *ALG7* expression results in diminished N-glycosylation and hypersensitivity to tunicamycin. More recently, Giaever *et al.*

described the increased sensitivity of an *ALG7/alg7* heterozygous diploid *S. cerevisiae* strain to tunicamycin. The analysis described below showed that when the gene which encodes the dolichol-P-dependent N-acetylglucosamine-1-P transferase (GPT) was down regulated by the presence of copper, sensitivity to tunicamycin was

5 increased. The studies demonstrate that lowering the dosage of an essential gene results in a population sensitized to compounds that acts on the product of that gene.

In a single 96 well assay plate, a serial dilution of copper sulfate was created from right to left. On the same plate a serial dilution of compound was created along the opposite axis to give the final compound and copper concentrations.

10 Each well was inoculated with  $10^3$  cells/mL. Yeast cell growth was scored by eye after 48 hours incubation at 37° C.

Plates were setup as described above and inoculated with wild-type, copper regulated *ALG7* and copper regulated *CHS3* strains. *CHS3* encodes chitin synthase and provides a second control. Following 48 hours incubation at 37° C,

15 plates were examined to assess growth (Figure 16). The studies showed that only the *ALG7* copper regulated cells demonstrated increased sensitivity to tunicamycin as the copper concentration was increased (Figure 16, panel a). No increase in sensitivity to tunicamycin was seen when non-target specific genes were down regulated, i.e. *CHS3* (Figure 16, panel b) or wild type (Figure 16, panel c) controls. These data

20 demonstrate that copper acts synergistically with the antifungal agent tunicamycin and that the increased tunicamycin sensitivity is due to a reduction of the *ALG7*-encoded target.

Other strains engineered to regulate previously identified as drug targets were also examined. The copper regulated gene, the function of the target protein, and

25 the drug tested are provided in the table below. The *ALG7* and tunicamycin experiment was repeated as a positive control.

GATE strain	Function of target	Compound
Cu <i>ERG11</i>	ergosterol synthesis	Azole
Cu <i>TUB1</i>	polymerization of tubulin	Benomyl
Cu <i>CMD1</i>	Ca <sup>2+</sup> regulation via calmodulin	Fluphenazine
Cu <i>ALG7</i>	N-Glycosylation	Tunicamycin

The results of this analysis are shown in Figure 17, panels a-d. In each case the strain demonstrated greater sensitivity to the compound as the copper concentration was increased. These experiments show that the strains of the invention can be used to identify target genes for antifungal compounds and provide tools for the analysis of antifungal agents and their mechanisms of action. Rapid reduction of antifungal target gene products lead to increased sensitivity to that agent and provide a phenotype by which to analyze the possible mechanisms by which the antifungal agents acts.

**Claims:**

1. A method for generating a yeast strain comprising a regulated target gene comprising:

(a) generating a yeast cell comprising

(i) a first gene encoding a transcriptional repressor protein whose expression is under the control of a metal ion-responsive element, wherein expression of said first gene encoding said repressor protein is stimulated by the addition of a metal ion to growth medium of said yeast cell;

(ii) a second gene encoding a subject protein, wherein expression of said second gene encoding said subject protein is controlled by a transcriptional control sequence whose activity is inhibited by said repressor protein; and

(iii) a third gene encoding a biomineralization protein, wherein said third gene is inactivated and wherein inactivation of said third gene enhances transcriptional response of said metal ion-responsive element to metal ions in said growth medium of said yeast cell;

(b) culturing the yeast cell in a growth medium comprising metal ions, wherein said metal ions are present in sufficient concentration to activate said metal ion-responsive element to a level which will result in said predetermined level of repression of expression of said subject gene;

(c) assessing whether the rapid depletion of the second gene from the yeast cell leads to inhibition of cell growth or cell death.

2. The method of claim 1, wherein said inhibition identifies the target gene as an essential target gene.

3. The method of claim 1, wherein said transcriptional repressor protein is the protein encoded by the ROX1 gene.

4. The method of claim 1, wherein said transcriptional control sequence is ANB1 promoter.

- 1                   5.     The method of claim 1, wherein said biomineralization gene is SLF1.
- 1                   6.     The method of claim 1, wherein the yeast cell further comprises a fourth  
2 gene encoding a protein that targets ubiquitin-containing polypeptides for degradation.
- 1                   7.     The method of claim 6, wherein said fourth gene is placed under the  
2 control of a metal ion-responsive element.
- 1                   8.     The method of claim 6, wherein said fourth gene is UBR1.
- 1                   9.     The method of claim 1, wherein said transcriptional repressor protein is  
2 CYC8-LexA.
- 1                   10.    The method of claim 1, wherein said gene encoding said transcriptional  
2 repressor protein is ROX1-LexA.
- 1                   11.    A method of screening a candidate antifungal compound for interaction  
2 with an essential target gene comprising:  
3                   (a) generating a regulated yeast strain comprising a regulated essential  
4 target gene as defined in claim 1;  
5                   (b) establishing a concentration of metal ion at which the growth or  
6 viability of the regulated yeast strain ceases;  
7                   (c) generating a serial dilution of metal ion in yeast growth media;  
8                   (d) culturing the regulated yeast strain in the serially diluted growth media,  
9 wherein the serial dilution leads to a dose-dependent modulation of expression of the  
10 regulated essential target yeast gene product;  
11                   (e) screening the serially diluted cultures for altered sensitivity of the  
12 strain to the candidate antifungal compound;  
13                   (f) determining the metal ion concentration present in a culture  
14 demonstrating altered sensitivity to the candidate antifungal compound;  
15                   (g) comparing the metal ion concentration of step (b) with the metal ion  
16 concentration of the culture determined in step (f), and identifying a candidate antifungal

7 compound for which a lower concentration of metal ion is required to eliminate growth  
8 or viability in step (f) as compared to step (b).

1 12. The method of claim 11, wherein a plurality of candidate antifungal  
2 compounds are screened.

1 13. The method of claim 12, wherein the screen of the plurality of candidates  
2 is selected from the group consisting of a screening together in a single assay and a  
3 screening individually using multiple simultaneous individual detecting steps.

1 14. The method of claim 11, wherein said transcriptional repressor protein is  
2 the protein encoded by the ROX1 gene.

1 15. The method of claim 11, wherein said transcriptional control sequence is  
2 ANB1 promoter.

1 16. The method of claim 11, wherein said biomineralization gene is SLF1.

1 17. The method of claim 11, wherein the yeast cell further comprises a fourth  
2 gene encoding a protein that targets ubiquitin-containing polypeptides for degradation.

1 18. The method of claim 15, wherein said fourth gene is placed under the  
2 control of a metal ion-responsive element.

1 19. The method of claim 15, wherein said fourth gene is UBR1.

1 20. The method of claim 11, wherein said transcriptional repressor protein is  
2 CYC8-LexA.

1 21. The method of claim 11, wherein said gene encoding said transcriptional  
2 repressor protein is ROX1-LexA.

1           22.    A method of rapidly cloning a DNA complementary to an essential target  
2   gene comprising:

                  (a) generating a regulated yeast strain comprising a regulated essential  
target gene as defined in claim 1;

                  (b) establishing a concentration of metal ion at which the growth or  
6   viability of the regulated yeast strain ceases;

                  (c) transforming the regulated yeast strain with a DNA to be tested for  
complementation;

                  (d) culturing the transformed regulated yeast strain in growth media  
containing a concentration of metal ion as established in step (b);

11               (e) determining the ability of the DNA to complement the regulated  
essential target gene, wherein growth or viability of the regulated yeast strain establishes  
complementation; and

                  (f) cloning the complementary DNA.

1           23.    The method of claim 22, wherein the DNA is selected from the group  
2   consisting of genes from another organism, mutant DNA and DNA fragments.

3  
4           24.    The method of claim 23, wherein the organism is selected from the group  
5   consisting of human, mouse, mammal, drosophila and mycete.

1           25.    The method of claim 22, wherein the DNA is generated from the group  
2   consisting of genomic and cDNA libraries.

1           26.    The method of claim 22, wherein said transcriptional repressor protein is  
2   the protein encoded by the ROX1 gene.

1           27.    The method of claim 22, wherein said transcriptional control sequence is  
2   ANB1 promoter.

1           28.    The method of claim 22, wherein said biomineralization gene is SLF1.



1           29.    The method of claim 22, wherein the yeast cell further comprises a fourth  
2           gene encoding a protein that targets ubiquitin-containing polypeptides for degradation.

1           30.    The method of claim 29, wherein said fourth gene is placed under the  
2           control of a metal ion-responsive element.

1           31.    The method of claim 29, wherein said fourth gene is UBR1.

1           32.    The method of claim 22, wherein said transcriptional repressor protein is  
2           CYC8-LexA.

1           33.    The method of claim 22, wherein said gene encoding said transcriptional  
2           repressor protein is ROX1-LexA.

1           34.    A method of determining an antifungal effect of an antifungal compound  
2           comprising:

3                   (a) generating a regulated yeast strain comprising a regulated essential  
4           target gene as defined in claim 1;

5                   (b) establishing a concentration of metal ion at which the growth or  
6           viability of the regulated yeast strain ceases;

7                   (c) culturing the regulated yeast strain in growth media containing the  
8           concentration of metal ion as established in step (b);

9                   (d) determining the phenotype associated with the culture of step (c) that  
10          is depleted of the essential target gene;

11                  (e) culturing a yeast strain in growth media with a candidate antifungal  
12          compound;

13                  (f) determining the phenotype associated with the culture of step (e) that  
14          is treated with the candidate antifungal compound;

15                  (g) comparing the phenotypes determined in steps (d) and (f) to determine  
16          the antifungal effect of the antifungal compound.

1           35.    The method of claim 34, wherein determining the phenotypes comprises

- 2 (i) incubating the cultures with radio-labeled macromolecular  
3 building-blocks;  
4 (ii) establishing a level of incorporation of the radio-labeled  
5 macromolecular building blocks for each culture; and  
6 (iii) analyzing the macromolecular products generated in each  
7 culture.

1 36. The method of claim 34, wherein said transcriptional repressor protein is  
2 the protein encoded by the ROX1 gene.

37. The method of claim 34, wherein said transcriptional control sequence is  
ANB1 promoter.

1 38. The method of claim 34, wherein said biomineralization gene is SLF1.

1 39. The method of claim 34, wherein the yeast cell further comprises a fourth  
2 gene encoding a protein that targets ubiquitin-containing polypeptides for  
3 degradation.

1 40. The method of claim 39, wherein said fourth gene is placed under the  
2 control of a metal ion-responsive element.

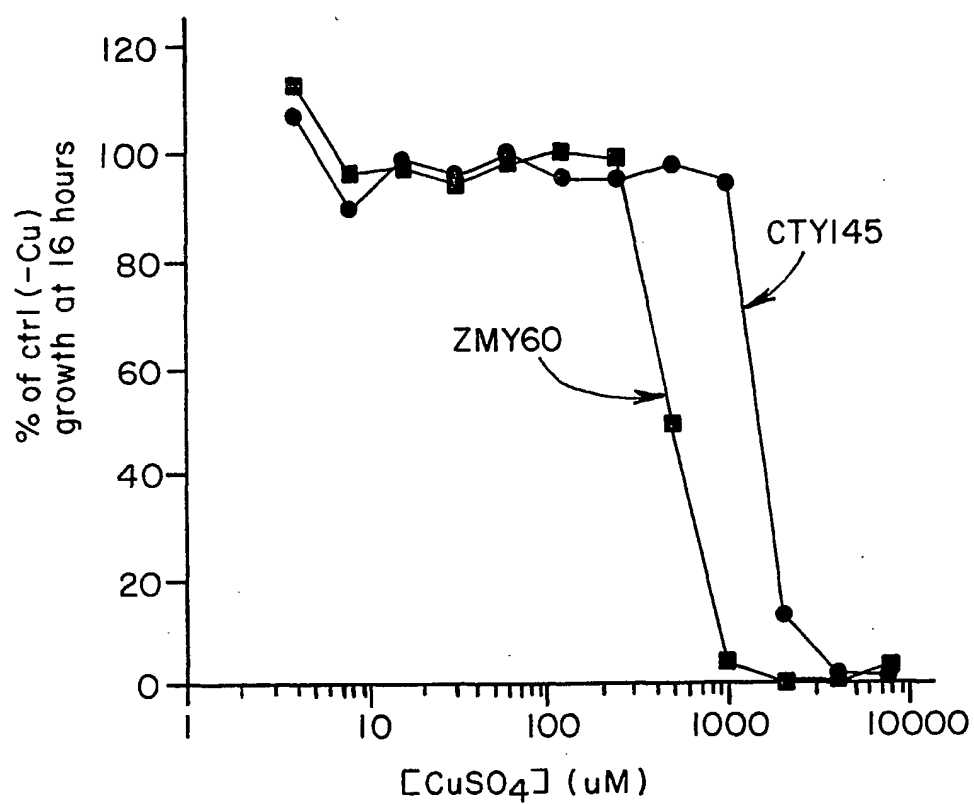
1 41. The method of claim 39, wherein said fourth gene is UBR1.

1 42. The method of claim 34, wherein said transcriptional repressor protein is  
2 CYC8-LexA.

43. The method of claim 34, wherein said gene encoding said transcriptional  
repressor protein is ROX1-LexA.

1/25

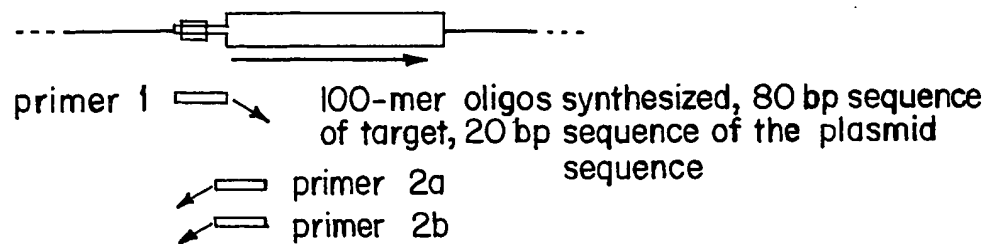
FIG. 1



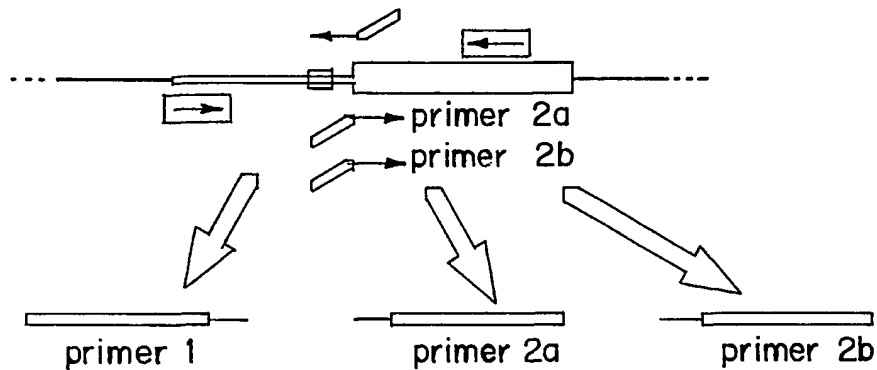
2/25

## FIG. 2A

### A. SINGLE ROUND PCR STRATEGY (OLIGOS SYNTHESIZED):



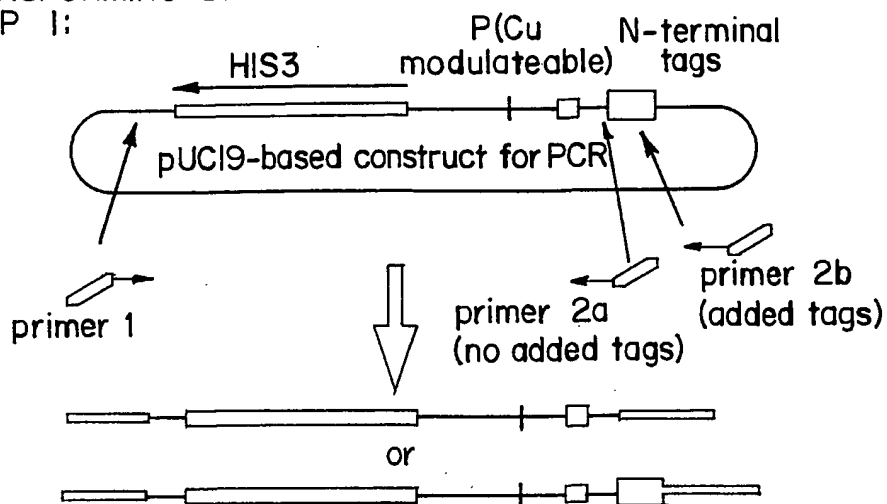
### B. DOUBLE ROUND PCR STRATEGY (OLIGOS PRODUCED BY PCR)



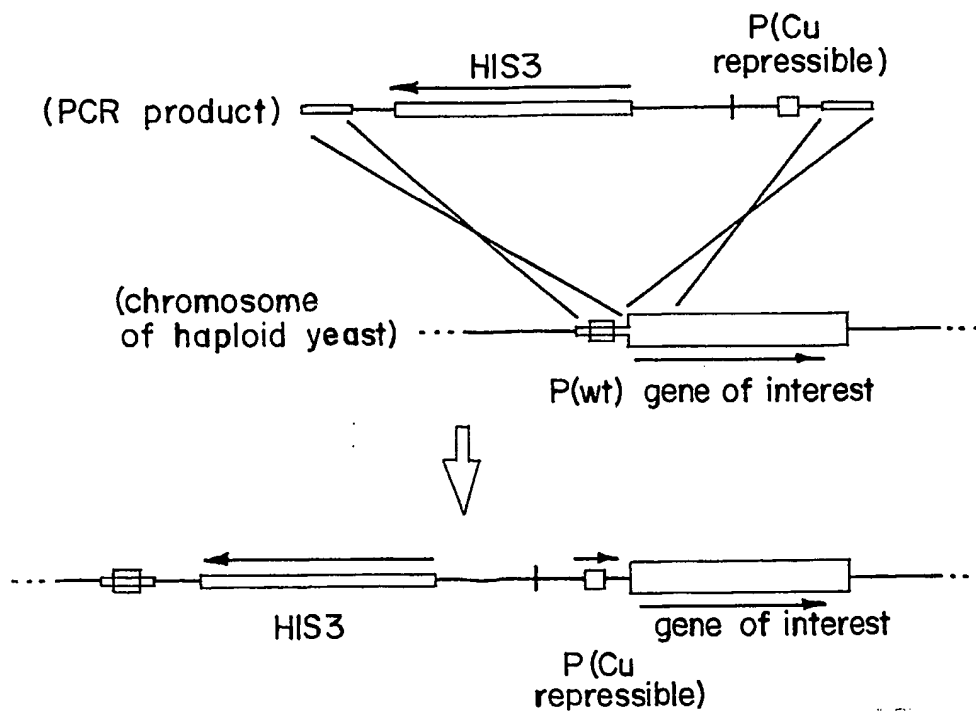
3/25

**FIG. 2B**

II: TRANSFORMING DNA PRODUCED BY PCR WITH OLIGOS FROM STEP I:

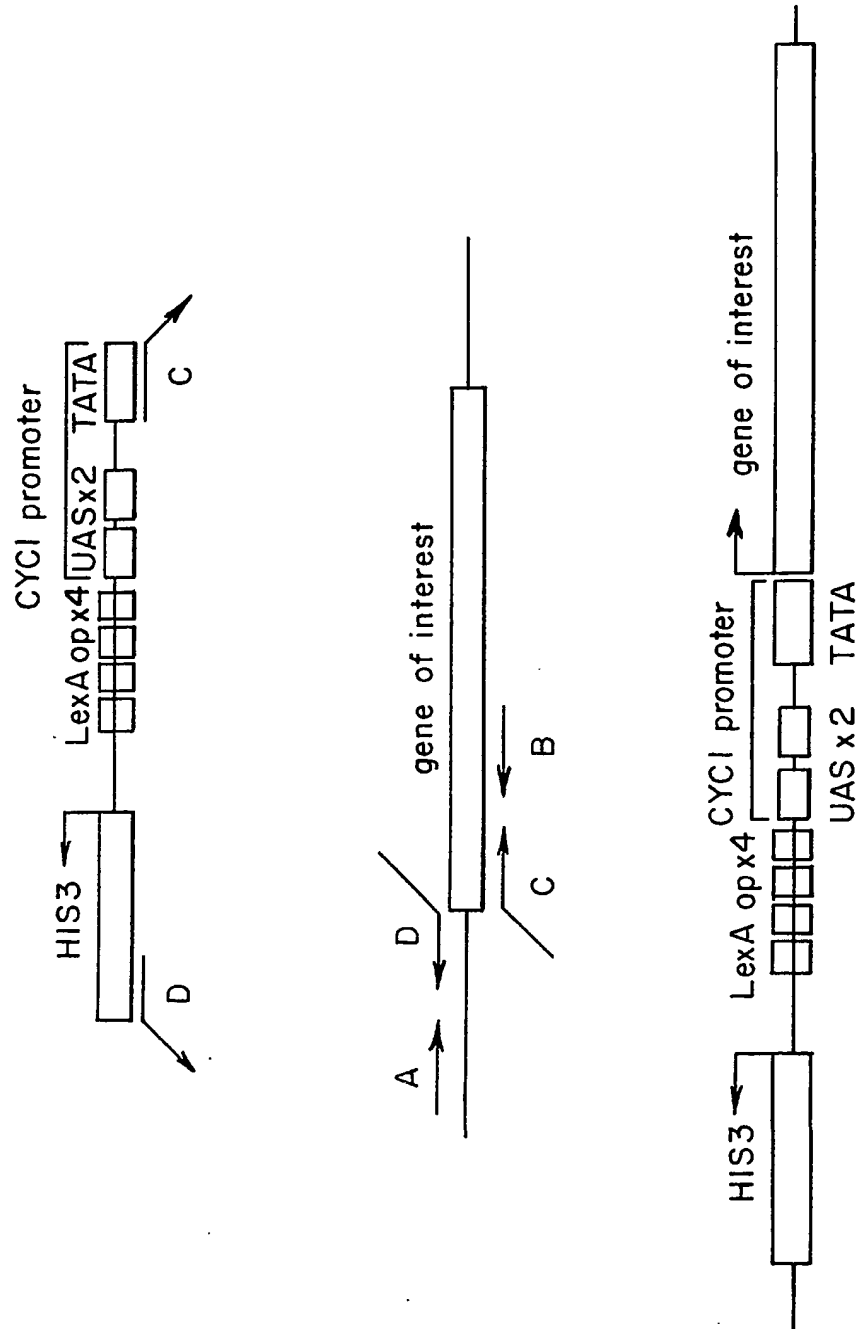
**FIG. 2C**

III: TRANSFORMATION, RECOMBINATION



4/25

FIG. 3



5/25

## FIG. 4A

GAATTAAATTCGAGTCGGTACCGGTGATCTTCGCTCGGCCACAAATCCCCTGGATATCATTTGGCC  
TGTCGAGGTATCGGCCGCTGGAACCTACCGGAATTACTATGCAAAACAATTGGAAATCTGGTAG  
GAAACCTTGTTCTAGAACTTGCGGATGCTGACAAAGAAAGGCCCTATTGTTGCTGCCCTC  
TTTTTGTGTTCTTCCCTCGTATTGCTTGGCGGTGTTCTTGTGTCCTTTTGTGTAGGTTCTTAC  
TATTATAGTGTCTTTTGCTATTATATTTTCTTCTGCTTTCACCTTTCGCTATCTTATTTGCTAATTGTAGT  
CAAAGTTTTTTTTTCTGCTCTTGCAATTTTCTTCTGCTTTCCTTAAATGTTTCTTCTCATTTGCTTCT  
TTCAGAAAGTTTTTACCTTAAATATAGCACTATTTTCCAGTTTAAATGTTTCTTCTCATTTGCTTCT  
TTTATAATTTTCGCATATAATATACATTTACGGTGTCTTAACTCTCCCTCTTCACCCCTCATTA  
TTCAGAAAATACATAACTTCTTACACAAAAGAACGAGTTAGACAAATCAACAATGACTAGTA  
GTTTTTCTTGAAACCAAGAAAGTCAACGAGGCAATAGACTCTTCAATCTCATTTGATTTCTTTGC  
TTGGCTTCTGCAGTGGACGAGAACTTGGCTTTTTCCTTAACTTCTCCCTCAATTTGGTTGTTTTT  
TCTCTTGATTTGAGCATCCAAATGCTTAAATAGAGTCGTGAATGTTGCTTCTACGGGTTTTCAAGT  
CAGCTTGGATCTTGATGATCTCTTGTCTTAACTCTGTAACCTTCTTACGTTCTTGTGTTGGTGGTA  
TCGTTGACCTGGTGTGATCGATTGCTTCTAAATTAACCGATTTCAGTGTGCAATTTTTCAA  
TTGAACGTTAAGAGTGTCCAAATTTCTTGTCTCTAACGGAGACATCTGGCGCTTGAACTTGTGTT  
GTTGGAGGACATGGCAATGGCTGTGTTGTTAGAAAATATGCTATTACGTTGATAAAAGGAGGAA  
AGGTGAAATCAGTTCAAAATGTGAATGAACTGAACGAGAAATGACCAGAAATGAGTGAAAAAT  
GGAGATGGAGGGCAAAATGAAAAAAGGATGAACCTAAAATAGAAAAATAGACTCCGTC  
GTACTTTAATGCTATGTATAACGCAACCAAGCAATTTTCGAACTCAATTTTGGCTTATAAATGTT  
CGAGATAAAATGCGAATTACGTGTTCAACGTCTCGAGATCAGTTATTTTTCACGCCACAGT  
GCGGTAAGCAATTTTTCGCGTACCAACCACTTACACATGTATAATGTAATAGGCTTATATG  
TATGTTTGTGCTACTTTATATGACGGTTATTTACAGTTAGAAATATCTATTAAACAATGCAGT  
AGCCACGCTTACGTTTAGTGAGTCAACAATGGGTTCTGGGGCCCGATTGCCCTTCTCAATGCCAC  
CAAAGGGAATTTTCGACGAAGAAGTCACCTCTCATCTTCAAAATTCGTTCTTACGCCCTGGCTTTCG  
TTCCCCACCACTAGAACCAACAGGCAGCTCGTTACATAATCCGTTCAAAATCGTGCAATGCTAATAGT  
TTTTCCCAACAGTGTATTTTTCTGACGTGGCATTAGCTAAGTGGCTGTATAAACCCTCCAGCCAC  
CCATTCTTGTGATTTAGTAAAAAACTTAACGGTTTATCAACGTAAAAATATGGGCAGAAAGTTTCG  
AGGGCCCCACTGCTTGTCTTGGACACCAACAGGCGTCAAAAGGAGAGCAGTTTCTTCTCGACATCAC

A

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## FIG. 4B

A  
AATGAAGTCAACCCAGGAAGTAAGCGCTTCTTAATAATGGCACCCGATATTGTGAGGTGAGTTA  
TTTCATCCAGATATAACCCGAGAGGAAACTTCTTAGCGTCTGTTTCGTACCATAAAGCAGTTCA  
TGAGGTATATTTTCGTTATTGAAGCCAGCTCGTGAATGCTTAATGCTGCTGAACCTGTTCCAT  
GTCGCCTAGGTACGCAATCTCCACAGGCTGCAAGGTTTGTCTCAAGAGCAATGTTATTGTGCA  
CCCCGTAAATTGGTCAACAAGTTTAATCTGTGCTTGTCCACCAGCTCTGTCTGTAACCTTCAGTTCA  
TCGACTATCTGAAGAAATTTACTAGGAATAGTCCCATGGTACAGCAACCGAGAAATGGCAATTTCT  
ACTCGGGTTCAGCAACGCTGCATAAACGCTGTTGGTCCGTAGACATATTCGAAAGATAGGATTAT  
CATTCATAAGTTTCAGAGCAATGTCCTTATTCTGGAACTTGGATTATGGCTCTTTTGGTTTAAT  
TTCCGCTGATTCTTGATCTCCTTTAGCTTCTCGACGTGGGCTTTTCTTCCATATGGATCTGA  
ATTCAGTCTTTTGTGGAACGTTGAGCGGAAAGACGCATCGAATTCGAGCTCGTTAGCGA  
TTGGCATTATCACATAATGAATTATACATTATATAAGTAATGTGATTTCTTCGAAGAATATACT  
AAAAATGAGCAGGCAAGATAAACGAAGCAAGACGGTATCGATATCAATGAATCCTAAATCC  
TCTACACCTAAGATTCCAAGACCCAGAACGCTTATTCTGTTCAGACAGCACTACCAAGGAT  
CTTAATAGACGAATGGACCGCTCAAGGTGTGGAAATACCCCATAAATCAAACTTTCTAAATTA  
TTGGTACGAAGTGGAGGGCTTACAAACCGGAAGATAAGGCACACTGGGAAATCTAGCGGAGAG  
GAGAACTAGAACATGAAGGAAGTATCTGTAATACAAATACAAAGCCGTAAGAAAGCTAAGAA  
GAAGCAACTACTTTTGAAGGAATCGAGCAACAGCAGCAACACAGAAAGAACAGCAGCAGC  
AGAAACAGTCAACAACCGCAATTACAAACAGCCCTTTAAACAACATATAGTTCTTATGAAAAGAGCA  
CATTCCTTTTCAACCATCTTCCGCTGTCAAGCTCGAACAGCTATCAGTTCCAAATGAACAAATGA  
TCTTAAGAGGTTGCCCTATTCTCTCTGTTAATACTTCTAACTATATGGTCTCCAGATCCTCTAGAG  
TCGACCTGCAGGCATGCAAGCTTGGCGTAATCATGTCATAGCTGTTTCCGTGTGAAATTTGTTA  
TCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGTGCTAAT  
GAGTGAGCTAACTCACATTAATTGCGTTGCGTCACTGCCGCTTCCAGTCGGGAACCTGTGCG  
TGCCAGGGGGATCCACTAGTTCTAGAGTCGACCGGCATGCAAGCTTGGCGTAATCATGTGCTATA  
GCTGTTTCTGTGTGAAATTGTTATCCGCTCAAAATTCACACAACATACGAGCCGGAAGCATAA  
AGTGTAAGCCTGGGGTGCCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCC

B



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## FIG. 4C

B

GCTTTCAGTCGGGAAACCTGTGTCGCCAGCTGCATTAATGAATCGGCCAAACGCCGGGAGAGG  
CGTTTTCGCTATTGGGCGCTCTTCCGCTTCTCCGCTCACTGACTCGTCGCTCGGTCTCGTTCGGC  
TGCGGCGAGCGGTATCAGCTCACTCAAAGCGGTAATA CGGTTATCCACAGAAATCAGGGGATAAC  
GCAGGAAAAGAAATGTGAGCAAAAGCCAGCAAAAGCCAGGAAACCGTAAAAGCCCGTTGCT  
GGCGTTTTCATAGGCTCCGCCCCCTGACGAGCATCAAAAAATCGACGCTCAAGTCAGAGGT  
GGCGAAACCCGACAGGACTATAAAGATACCCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGGCTCT  
CCTGTTCCGACCCCTGCCGCTTACCGGATACCTGTCCGCTTCTCCCTTCGGGAAGCGTGGCGCT  
TTCTCATAGCTCAGCTGAGGTATCTCAGTTCGGTGTAGTTCGTTTCGCTCCAAGCTGGGCTGTG  
TGACGAAACCCCGTTACGCCGACCGCTGCCCTTATCCGGTAACTATCGTCTTGAGTCCAAC  
CCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAAACAGGATTAGCAGAGCGAGGTA  
TGTAGGCGGTGTACAGAGTTCTTGAAGTGGTGGCTAACTACGGCTACACTAGAAGGACAGTAT  
TTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGC  
AAACAAACCAACCGTGGTAGCGGTGTTTTCGTTTGTGCAAGCAGCAGATTACCGCAGAAAAAA  
AGGATCTCAAGAAGATCCTTTGATCTTTCTACGGGCTGTGACGCTCAGTGAACGAAAACTCAC  
GTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACTAGATCTTTTAAATTAAAAA  
TGAAGTTTAAATCAATCTAAAGTATATATAGTAAACTTGGTCTGACAGTTACCAATGCTTAAT  
CAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTGCCCTGACTCCCCGTCG  
TGTAGATAACTACGATACGGGAGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGAC  
CCACGCTCACCGGCTCCAGATTATCAGCAATAAACCAGCCAGCCGGAAGGCCGAGCGCAGAAG  
TGGTCCCTGCAACTTATCCGCTCCATCCAGTCTATTAATGTTGCCGGAAGCTAGAGTAAGTA  
GTTCCGCCAGTTAATAGTTTGGCAACGTTGTTGCCATTGCTACAGGCATCGTGTGTCACGCTCG  
TCGTTTGGTATGGCTTCATTACGCTCCGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCAT  
GTTGTGCAAAAAGCGGTAGCTCCTTCGGTCCCTCCGATCGTTGTCAAGAAAGTTGGCCGCGAG  
TGTTATCACTCATGGTTATGGCAGCACTGCATAAATCTCTTACTGTCAATGCCATCCGTAAGATGC  
TTTTCTGTGACTGGTGAGTACTCAACCAAGTCAATCTGAGAAATAGTGTATCGGCGACCCGAGTTG  
CTCTTGCCCGCGTCAATACGGGATAATACCGGCCACATAGCAGAACTTTAAAGTGTCTCATCA  
TTGGAAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATG  
TAACCCCACTCGTGCAACCCAACTGATCTTCAGCATCTTTTACTTTCACCCAGCGTTTCTGGGTGAGC

C

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## FIG. 4D

C  
AAAAACAGGAAGCAAAATGCCGCAAAAAGGAATAAGGGCGACACGGAAAATGTTGAATACTCA  
TACTCTTCCCTTTTCAATATATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATA  
TTTGAATGTATTTAGAAAAATAAACAAATAGGGTTCGCGCACATTTCCCGAAAAGTGCCACC  
TGACGCTAAAGAAACCAATTAATCATGACATTAACTATAAAAATAGGCGTATCACGAGGCCAG  
CTTTCAATTCAATTCAATCATCTTTTCTTTTATTTCTTTTGTGATTTCCGTTTCTTTGAAATTTT  
TTTGATTCGGTAATCTCCGACAGAAAGAAACGAAAGGAGGACACAGACTTAGATTGGTATA  
TATACGCATATGTAGTGTGAAGAAAATGAAATTTGCCAGTATTCTTAACCCCAACTGCACAGAA  
CAAAAACATGCAGGAAAACGAGATAAATCATGTGAAAGCTACATATAAGGAACTGTGCTACT  
CATCCTAGTCTGTGCTGCCAAGCTATTTAATAATCATGCACGAAAAGCAAACTTGTGTGC  
TTCATTGGATGTTCTGTACCAAGGAAATTAATGAGTTAGTTGAAAGCATTAGGTCCCAAAATTT  
GTTTACTAAAAACACATGTGGATATCTTGACTGATTTTCCATGGAGGACAGTTAAGCCGCTA  
AAGGCATTAATCCGCCAAAGTACAAATTTTACTCTTCGAAGACAGAAAATTTGCTGACATTTGGTAA  
TACAGTCAAAATTCAGTACTCTGCGGTGTATACAGAAATAGCAGAAATGGCAGACATTACGAATG  
CACACGTGTGGTGGCCAGGTATTTGTAGCGTTTGAAGCAGGCGGACAGAAAGTAAACAAAG  
GAACCTAGAGGCCCTTTTGTAGTGTAGCAGAAATGTCTATGCAAGGCTCCCTATCTACTGGAGAATA  
TACTAAGGGTACTGTTGACATTCGGAAGAGCGACAAAGATTTTGTATCGGCTTTATTGCTCAAA  
GAGACATGGTGGAGAGATGAAGTTACGATTGGTTGATTAAGACACCCGGTGTGGTTTAGAT  
GACAAGGAGACGCATTGGGTCAACAGTATAGAACCCGTGGATGATGTGCTCTACAGGATCTGA  
CATTAATATTGTTGAAGAGGACTATTTCGAAAGGGAAGGATGCTAAGGTAGAGGTGAACGTT  
ACAGAAAAGCAGGCTGGGAAGCATATTGAGAAAGATCGGCCACAGCAAACTAAAAAACTGTATTA  
TAAGTAAATGCATGTATACTAACTCAAAATTAGAGCTTCAATTTAATATATCAGTTATTACC  
CGCCCTTTCGCTCGCGGCTTTCGGTGTATGACGGTGAACCTCTGACACATGCAGCTCCCGGAG  
ACGGTCAACAGCTTGTCTGTAGCGGATTCGCGGAGCAGACAAAGCCGTAGGGCGGTACGCGGG  
TGTTGGCGGTGTTCGGGCTGGCTTAATACTATCGGCGATCAGAGCAGATTGTACTGAGAGTGCACC  
ATATGCGGTGTGAATACCGCACAGATCGGTAAGGAGAAAATACCGCATCAGGCGCCATTGCGCA  
TTCAGGCTGCGCAACTGTTGGGAAGGCGATCGGTGCGGGCTCTTCGCTATTACGCCAGCTGGC  
GAAAGGGGATGTGCTGCAAGCGGATTAAGTTGGGTAAACGCCAGGGTTTCCCAGTCACGACGTT  
GTAAAAACGACGGCCAGT

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## FIG. 5A

GAATTAAATTCGAGCTCGGTACCAAGTTGCCACACCAAAAAGTCGAAAAAGGCTAAGAAACCAAAAGAAATAA  
GGTACTAAGTACCCAGGCGCTACTAAGACCAACGAGATTGCCACGAAACTAGAGGAAACCAAAATGTAAAG  
CATAGCTTAATCCGTTTTCACGATTCTATAATAATAAATAAGAAAAAGATATATCATATAAAACGTTATAA  
AATTAAATACCGGTAAGTGTAGAAAAGTGTGCGACGGTTTATTTTCTCTCTCCCTCTGCGATTGAATTT  
AACTTGCAGATAGTGACCATAAAGGCAACTACCCAGTGGCAACACAGTTTGTGATAAACGCCCAGTACATCAAC  
GAGCGAGTATAAAGACTTTGGTACATTTTAAAAAGGAAACATATATTTGTTTTCATTGCTAGACCCCTTTTA  
GTCTCACCTCAATAAAAATCTGCTTTATTTCTCATTTGGGCTTTTATTTCTTTAATTTTGCATACCTATAGCG  
TGAAACTGGGCATTTAACAAAAGCAAACTATTTTAAATAGTAGCATCCTGCTTTTCTTGGACAAAGGCAGT  
ATTGCGATACATTAATAAGTTTTTTACCACCTTTTCTCTCACTGAAGTCCCTAACTTTTACAGGTCACACA  
TGAAAGTTTACTGTATCCTATTAGTTGACTATTTTCTGAAACCAAGAAAGGTACCAAGGCAATAGACTCT  
AATTACATAGAACATTCCAACTAGTAGTTTCTTGAACCAAGAAAGGTACCAAGGCAATAGACTCT  
TCAATCTCATTGATTCTTTGCTTGGCTTCTGCAGTGGACGAGAACTTGGCTTTTGGCTTAACTTCTTCTT  
CAATTGCTGTTGTTTCTCTTGATTGAGCATCCAATTGCTTAAATAGAGTCGTGAATGTTGCTTCTTGTG  
GGTTTCAAGTCAGCTGGATCTTGATGATCTCCTTGTCTTATCCTGTAACCTTCTTACGTTCTTGTG  
GTGGTATCGTTGACCTGGTGTGATCGATTGCTTTCTAAATAAACCGATTTCACTGTCGATTTTCTTCA  
ATTGAACGTTAAGAGTGTCCAATTCTTGTCTCTAACGGAGACATCTGGGCGCTTGAACCTTGTGTTG  
GGAGGACATGGCAATGGCTGTGTTGTTAGAAAATATGCTATTACGTTGATAAAAGGAGGAAAGGTGAAAT  
CAGTTCAAAAATGTGAATGAAACTGAACGAAAGAAATGACCAAGAAATGAGTGAATAATGGAGATGGAGGGC  
AAAATGAAAAAAGGATGAACCTAAAATAGAAAATAGACTCCGTCGTACTTTAATGCTATGTAT  
AACGCAACCAAGCAATTTTCGAAACTCAATTTGGCTTATAAATGTTTCGAGATAAAATGCGAATTACGTGT  
TCAACGTCGTCGAGATCAGTTATTTTTCACGCCACAGTGGGTAAGCAATTTTTCGCGTACCA  
CCATTACACATGTATAATAGGCTTATATGTATGTTTGTGCTACTTTATATGACGGTTATTAC  
AAGTTAGAATAATATCTAATAACAATGCAGTAGCCACGCTTACGTTTAGTGAGTCAACAATGGGTTCTGG  
GGCCGATTGCTTCTCAATGCCACCAAGGGAATTTTCGACGAAGAGTCACTCCCTCATCTTCAAAATC  
GTTCTTACGCCCTGGCTTTCGTTCCCAACCACTAGAAACCAAGGAGCTCGTTACATAATCCGTTCAAAAT  
CGTGCATGCTAATAGTTTTCCAACAGTGTATTTTCTGACGTGGCATTAGCTAAGTGGCTTGTAAATAA  
CGTCCAGCCACCCATTTCTTGTGATTTAGTAAAAAACTCTAACGGTTTATCAACGTAAAAATATGGGCAGA  
AGTTCGAGGGGCCCCACTGCTTGTCTTGGACACCAAGGCGTCAAGGAGAGCAGTTTCTTCTCGACATCA

A

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## FIG. 5B

A  
CAATGAAGTCAACCCAGGAAGTAAGCGCTTCTAATAATGGCACCGGATATTTGTAGGGTCAGTTATTTTC  
ATCCAGATATAACCCGAGAGGAAACTTCTTAGCGTCTGTTTTCGTACCATAGGCAGTTTCATGAGGTATA  
TTTTTCGTTATTGAAGCCAGCTCGTGAATGCTTAATGCTGCTGAACCTGGTGTCCATGTCCGCTAGGTACG  
CAATCTCCACAGGCTGCAAGGTTTTGTCTCAAGAGCAATGTTATTGTGCAACCCCGTAATTGGTCAACAA  
GTTTAACTCTGTGCTGTGCCACAGCTCTGTGTAACCTTCAGTTTCATCGACTATCTGAAGAAATTTACTA  
GGAATAGTGCCATGGTACAGCAACCGAGAAATGGCAATTTCTACTCGGTTTCAGCAACGCTGCATAAACGC  
TGTTGGTGCCGTAGACATATTCGAAGATAGGATTTATCATTCATAAGTTTCAGAGCAATGTCCTTATTTCTG  
GAACTTGGATTATGGCTCTTTTGGTTTAAATTCGCCCTGATTTCTTGATCTCCTTTAGCTTCTCGACGTGG  
GCCTTTCTTCCCATATGGATCTGAATCTTAGTCTTTTTCGTGGAACGTTGAGCGGAAAGACGCAT  
CGAATTCGAGCTCGTTAGCGATTGGCATTTACATAAATGAATATACATATATAAAGTAATGTGATTT  
CTTCGAAGAAATATACTAAATAATGAGCAGGCAAGATAAACGAAGCAAGGACGGTATCGATAAGCTTGG  
GAAATCAAAATGCCCAAGAAAGCGGAAGGTCCATATGTACCCATACGACGTTCCAGACTACGCTTCTT  
TGGGTGTTCTAGCCCAAGCTTGATATCGAATTCCTGCAGCCCGGGGATCCTAACATGTCCGTTGCTGA  
TGATGATTTAGGATCTTTACAAGGTCACATTAGGAGAACACTGAGGTCTATTTCATAACCTCCCTATTTT  
AGGTATACGAGAGGTCCTACTGAAAGGCTGACATGAGCAGAGCCCTTAAAGAGTTTCATTACAGATATC  
TATACTTTGTCTATTCTAACAGCGGAGAGAACTTACCCTACTTTATTCAATGCTCATCCAAACAAAAAAT  
ATCTAACCCAGAGCTTACTGTTTTTCTTGACAGTTTAGAAGATGCTGTGGATATTGATAAGATAACATCT  
CAACAACTATTCCGTTTTTAAGATAGATGAATCCAGAAATAGGAGACGTCCTCAATAACATACCGGAAGAA  
ATTGFGGAGGAAATTCAAAATAGGGGAACCTTGTAATAGGTGTCTAGTGTGGTTGCCATGATACCTTG  
TGTGCTTTGTATTCTATTGTTTAAATCCAAAAGATCATGTGAATCATCATGTTTGTACCGATATATGTACT  
GAATTCGATATCAAGCTTGCGTAAATCATGGTCAATAGCTGTTTCCCTGTGTGAATTTGTTATCCGCTCACA  
ATTCACACAAATACGAGCCGGAAGCATAAAGTAAAGCCTGGGTGCCATAATGAGTGAGCTAACTCA  
CATTAATTGCGTTGCGCTCACTGCCCGTTTTCCAGTCGGGAAACCTGTCTGCCAGGGGGATCCACTAG  
TTCTAGAGTCGACCGGCAATGCAAGCTTGGCGTAAATCATGGTCAATAGCTGTTTCTGTGTGAATTTGTTAT  
CCGCTCACAATTCACACAAATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGTGCCCTAATGAGTGA  
GCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCTGTCAGCTGCA

B

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## FIG. 5C

B B

TTAATGAATCGGCCAACGCGCGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCCTCGCTCACT  
GACTCGTCGCTCGGTCGTTCCGCTGCGGCGAGCGGTATCAGCTCACTCAAAGCGCGTAATACGGTTAT  
CCACAGAAATCAGGGGATAACGACAGGAAACATGTGAGCAAAAGCCAGCAAAAGCCAGGAACCGTAA  
AAGGCCGCTTGCTGGCGTTTTCATAGGCTCCGCCCTGACGAGCATCACAAAATCGACGCTCA  
AGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGC  
GCTCTCCTGTTCCGACCTGCCGCTTACCGGATACCTGTCCGCCCTTCTCCCTTCGGGAAGCGTGGCGCT  
TTCTCATAGCTACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCCGCTCCAAGCTGGGCTGTGTGCAC  
GAACCCCGCTTCAGCCCGACCGCTGCCCTTATCCGTTAACTATCGTCTTGAGTCCAACCCCGTAAAGAC  
ACGACTTATCGCCACTGGCAGCAGCCACTGGTAAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTAC  
AGAGTTCTTGAAAGTGGTGGCTAACTACGGCTACACTAGAAGACAGTATTTGGTATCTGCGCTCTGCTG  
AAGCCAGTTACCTTCGGAAACAAAGATTGGTAGCTCTTGATCCGGCAACAAACCCGCTGTTAGCGGTG  
GTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTC  
TACGGGCTCTGACGCTCAGTGGAAACGAAACCTCACGTTAAGGATTTTGGTCATGAGATTATCAAAAAGG  
ATCTTCACCTAGATCCTTTTAAATTAATAATGAAGTTTAAATCAATCTAAAGTATATATAGTAAACTT  
GGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCAT  
AGTTGCCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGCTTACCATCTGGCCCCAGTGTGCA  
ATGATACCGGAGACCCACGCTCACCGGCTCCAGATTATCAGCAATAAACAGCAGCCAGCGAAGGCCCG  
AGCGCAGAAAGTGTCTGTCAACTTTATCCGCCCTCCATCCAGTCTATTAAATTGTTGCCGGAGCTAGAGT  
AAGTAGTTCCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGTGTACGCTCG  
TCGTTTGGTATGGCTTCATTAGCTCCGTTCCCAACGATCAAGCGAGTACATGATCCCCCATGTTGT  
GCAAAAAGCGGTTAGCTCCTTCGGTCCCTCCGATCGTTGTGAGAAAGTAAAGTGGCCGAGTGTATCACT  
CATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCCATCCGTAAAGATGCTTTCTGTGACTGGT  
GAGTACTCAACCAAGTCACTTGAGAAATAGTGTATGCGGCGACCGAGTTGCTCTTGGCCGGCTCAATAC  
GGATAATAACCGCGCCACATAGCAGAACTTTAAAGTGTCTCATCTATGGAAAACGTTCTTCGGGCGGAAA  
ACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAAACCCACTCGTGCAACCCAACTGATCTTCA  
GCATCTTTTACTTTACCCAGCGTTTCTGGGTGAGCAAAAACAGGAAGCAAAATGCCGCAAAAAGGGAA  
TAAGGGCGACACGGAAATGTTGAATACTCATCTCTCTTTTCAATATATTGAAGCATTTATCAGGG  
TTATTGTCTCATGAGCGGATACATAATTGAAATGTATTAGAAAAATAACAAATAGGGGTTCCCGGCACACA

C

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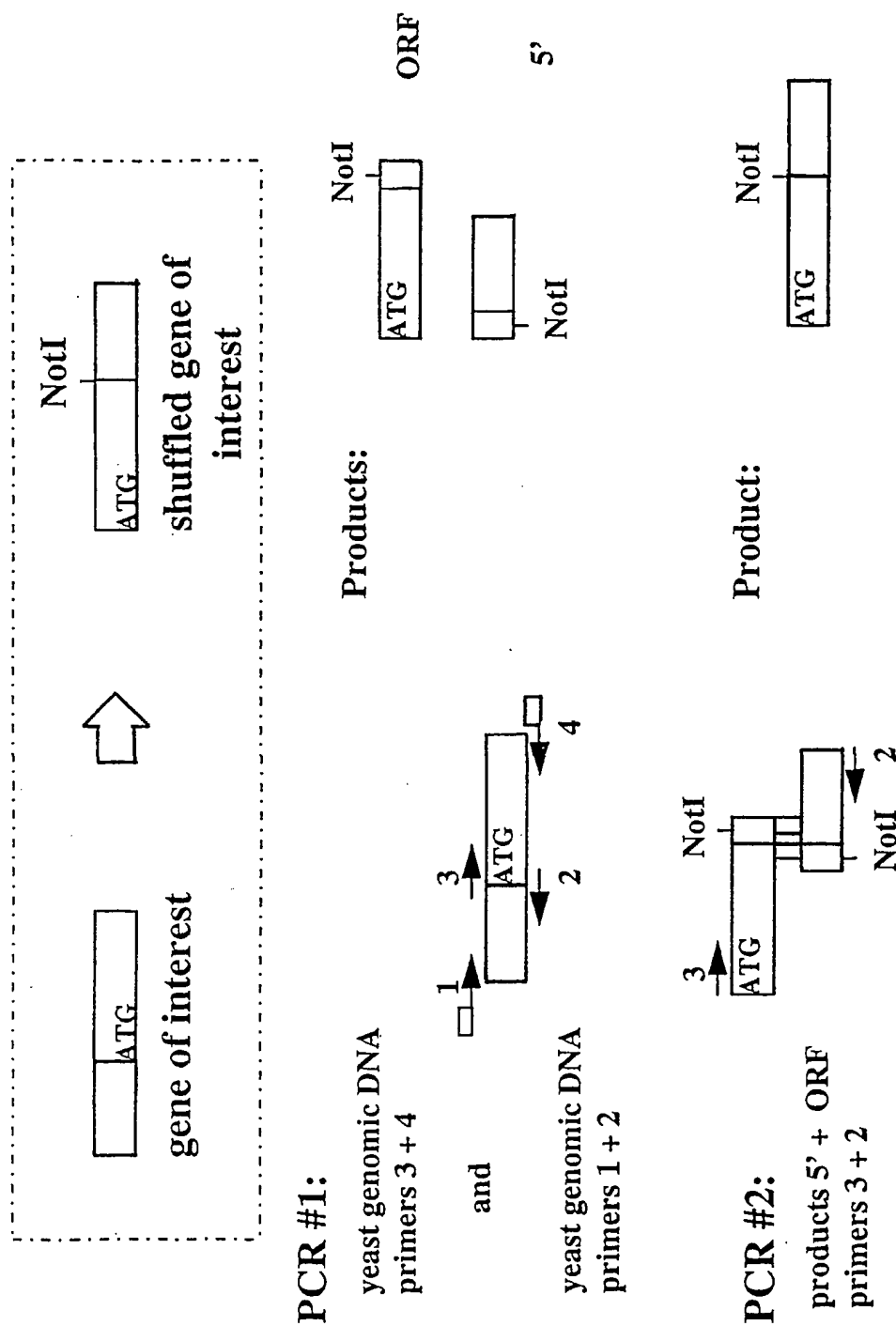
## FIG. 5D

C

TTTCCCGAAAAGTGCCACCTGCTAAGAAACCATTTATTATCATGACATTAAACCTATAAAAAATAGCGGTAT  
CAGAGCCAGCTTTTCAATTCAATTCAATCATCATTTTTTTTTTTTATTCTTTTGTGATTTTCGGTTTCTTTGA  
AATTTTGTGATTCGGTAAATCTCCGAACAGAAGAAACGAAGAGACACAGACTTAGATTGGTAT  
ATATACGCATATGTAGTGTGAAGAAACATGAATTGCCAGTATTCTTAAACCAACTGCACAGAAACAAA  
AACATGCAGGAAACGAAGATAAATCATGTGAAAGCTACATATAAGGAACGTGCTGCTACTCATCCTAGT  
CCTGTTGCTGCCAAGCTATTTAATATCATGCAGAAAGCAAAACAACTTGTGTGCTTTCATTGGATGTTTC  
GTACCAACCAAGGAATTACTGGAGTTAGTTGAAGCATTAGTCCCAAAATTTGTTTACTAAAAACACATGT  
GGATATCTTGACTGATTTTCCATGGAGGACAGTTAAGCCGCTAAAGGCATTATCCGCCAAGTACAAT  
TTTTTACTCTTCGAAGACAGAAAATTTGCTGACATTGGTAATAACAGTCAAAATTGCAGTACTCTGCGGGTG  
TATACAGAAATAGCAGAAATGGCAGACATTACGAATGCACACGGTGTGGTGGCCAGGTATTGTTAGCGG  
TTTGAAGCAGGCGGCAGAAAGTAACAAAGGAACCTAGAGGCCCTTTTGATGTTAGCAGAAATTGTCAATGC  
AAGGGCTCCCTATCTACTGGAGAATATACTAAGGGTACTGTTGACATTGCGAAGAGCGACAAGATTTTG  
TTATCGGCTTTATTGCTCAAAGAGACATGGGTGGAAGAGATGAAGGTTACGATTGGTTGATTATGACACC  
CGTGTGGGTTTAGATGACAAGGGAGACGCATTGGGTCAACAGTATAGAACCGTGGATGATGTGCTCT  
ACAGGATCTGACATTATTATTGTTGGAAGAGGACTATTTCGAAAGGGAAGGATGCTAAGGTAGAGGGTG  
AACGTTACAGAAAAGCAGGCTGGGAAGCATATTGAGAAGATGCGGCCAGCAAAACTAAAAACTGTATT  
ATAAGTAAATGCATGTATACATAACTCAAAAATTAGAGCTTCAATTATAATTATCAGTTATTACCCGCC  
CTTTCGTCTCGCGCTTTTCGGTGATGACCGTGAAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACA  
GCTTGTCTGTAAAGCGGATGCCGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGTGTTGGCGGGTGTGTC  
GGGCTGGCTTAATACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCAACCATATGCGGTGTGAAATACC  
GCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTTCGCCATTTCAGGCTGCGCAACTGTGTTGGAA  
GGCGATCGGTGCGGCCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGATGTGCTGCAAGGCCATTAA  
GTTGGGTAAACGCCAGGGTTTTCACAGTACGACGTTGTAAAAACGACGGCCAGT

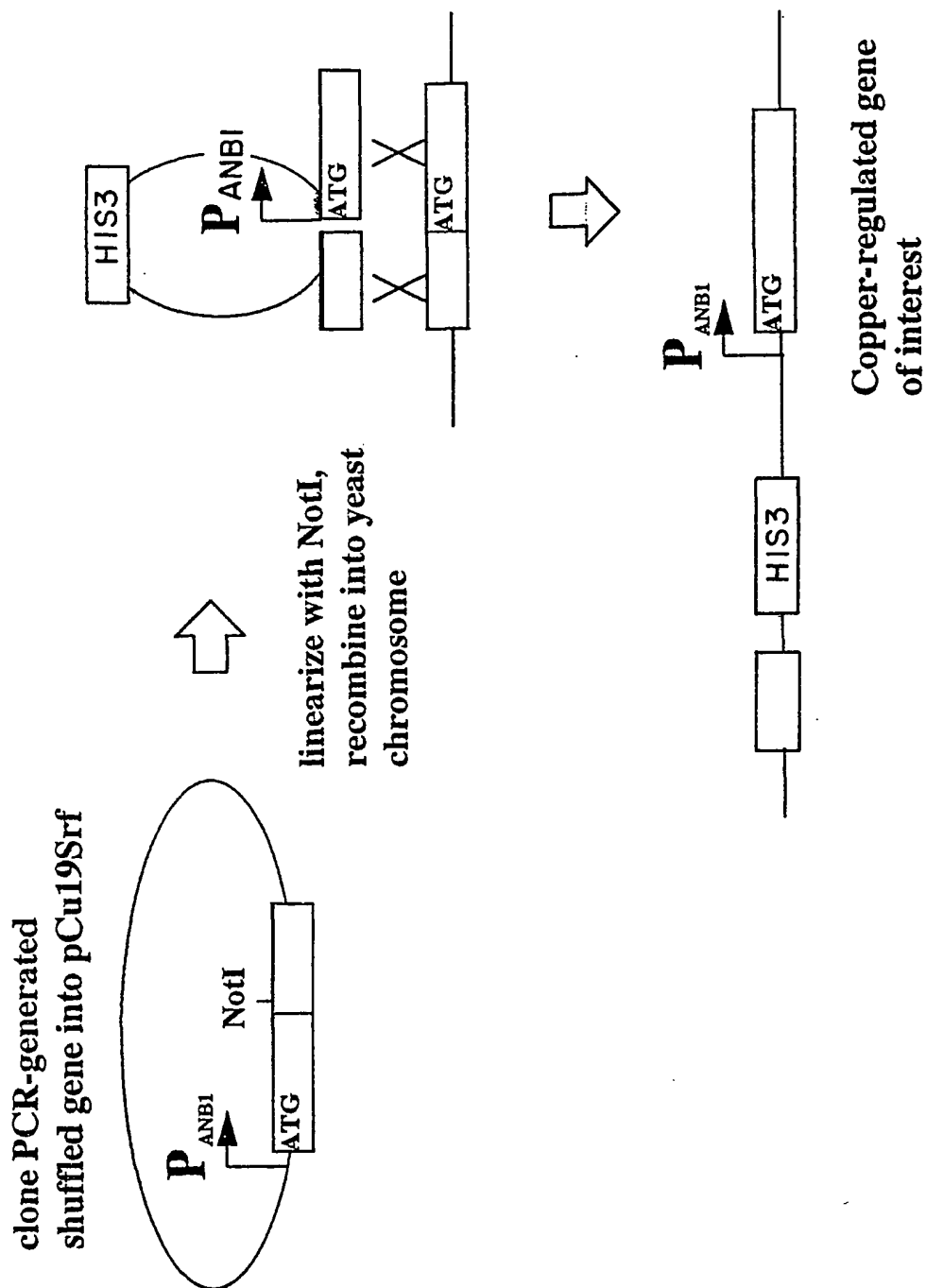
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FIG. 6



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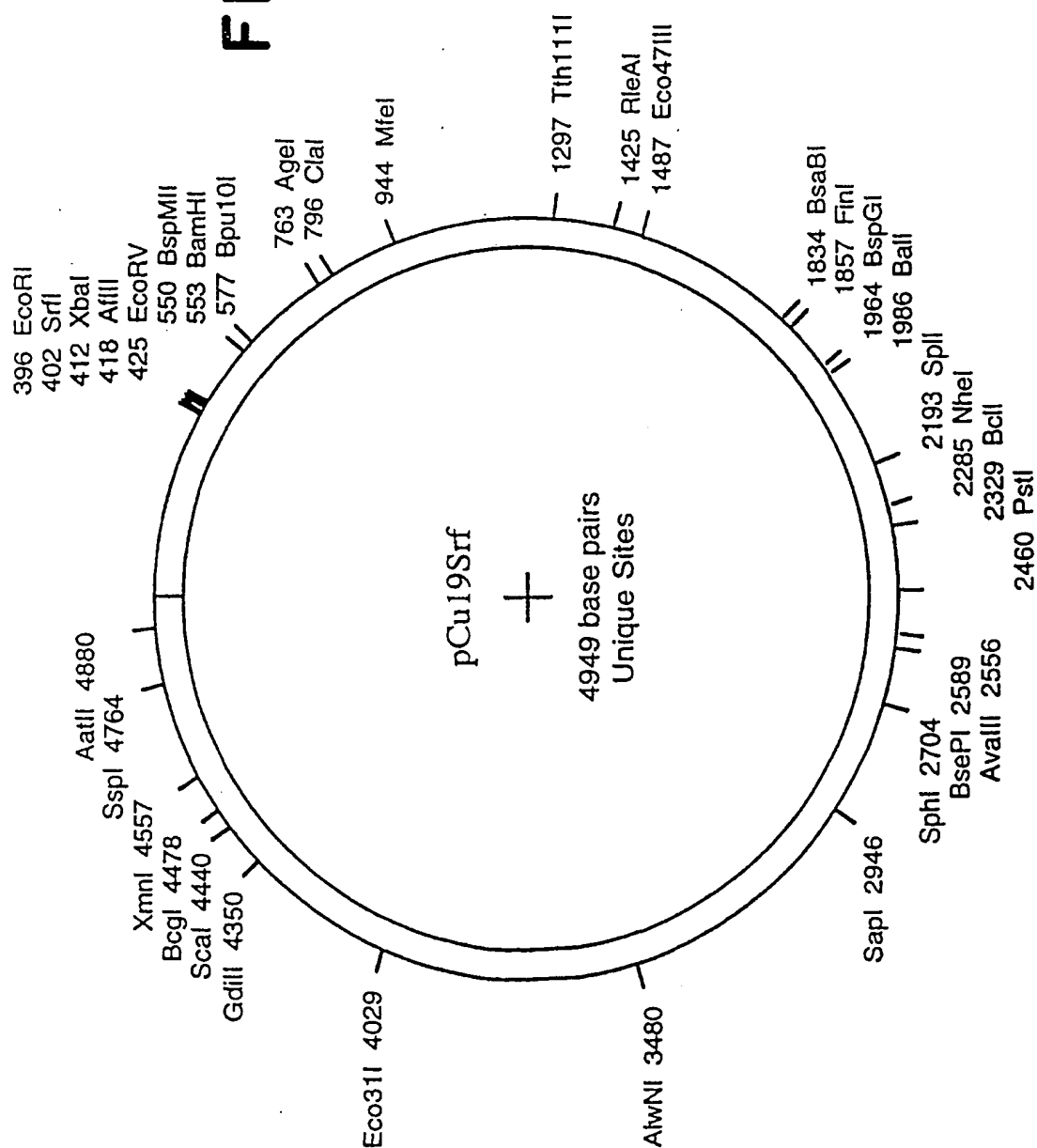
FIG. 7





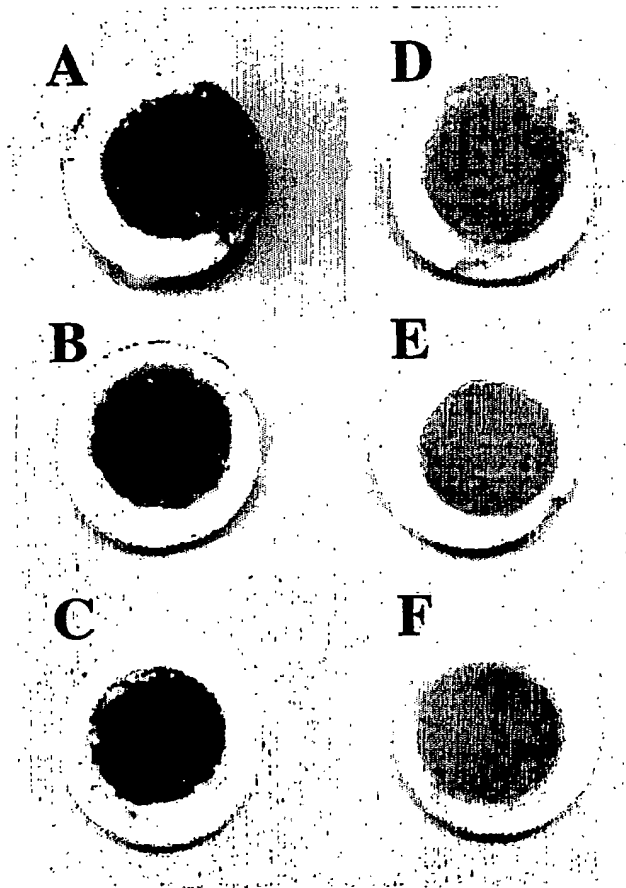
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FIG. 8



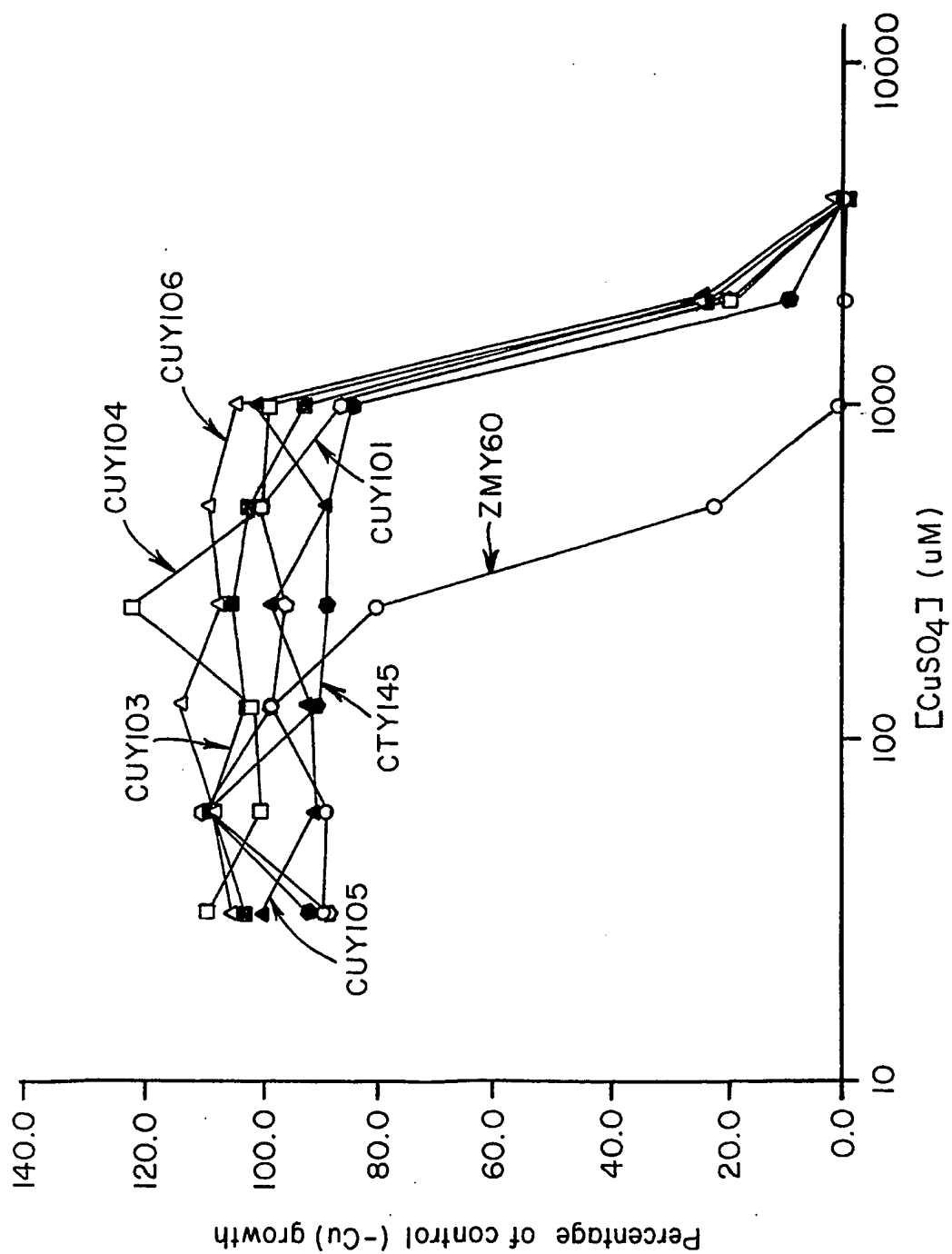
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**FIG. 9**



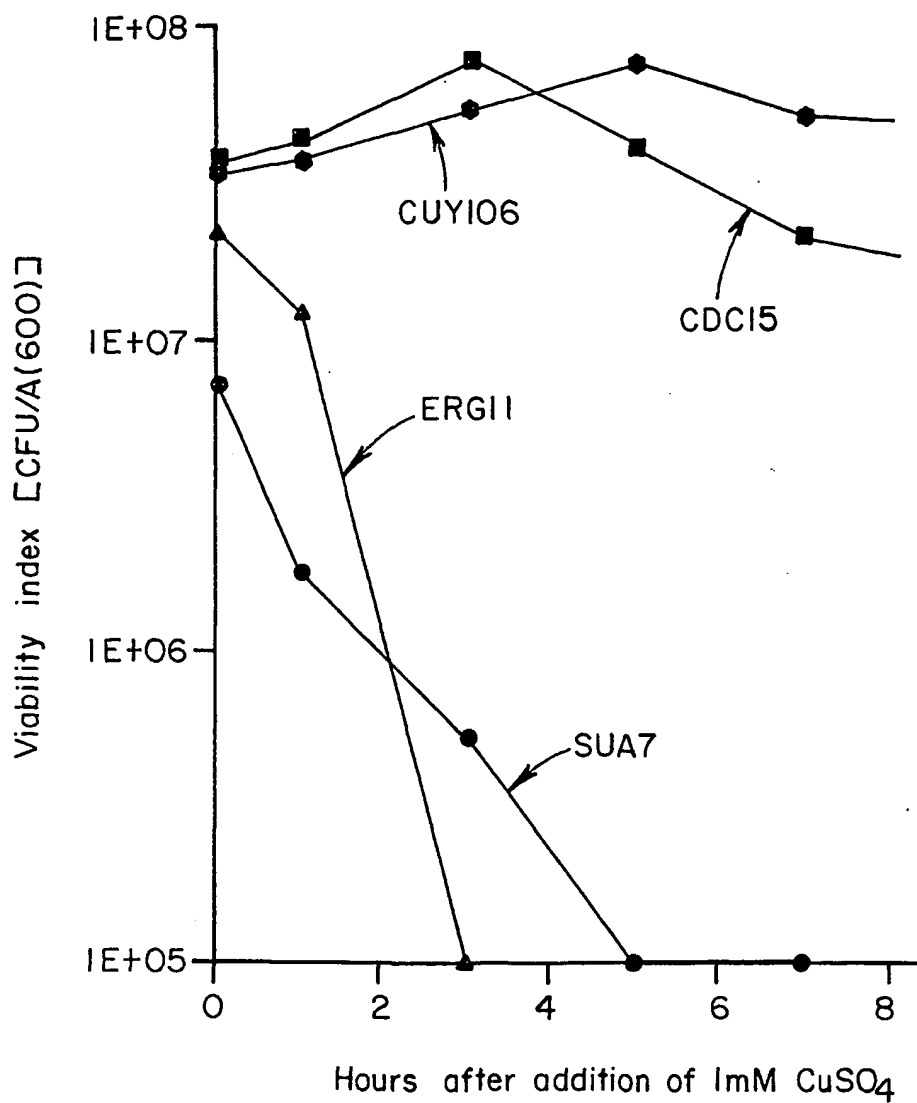
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FIG. 10



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FIG. 11



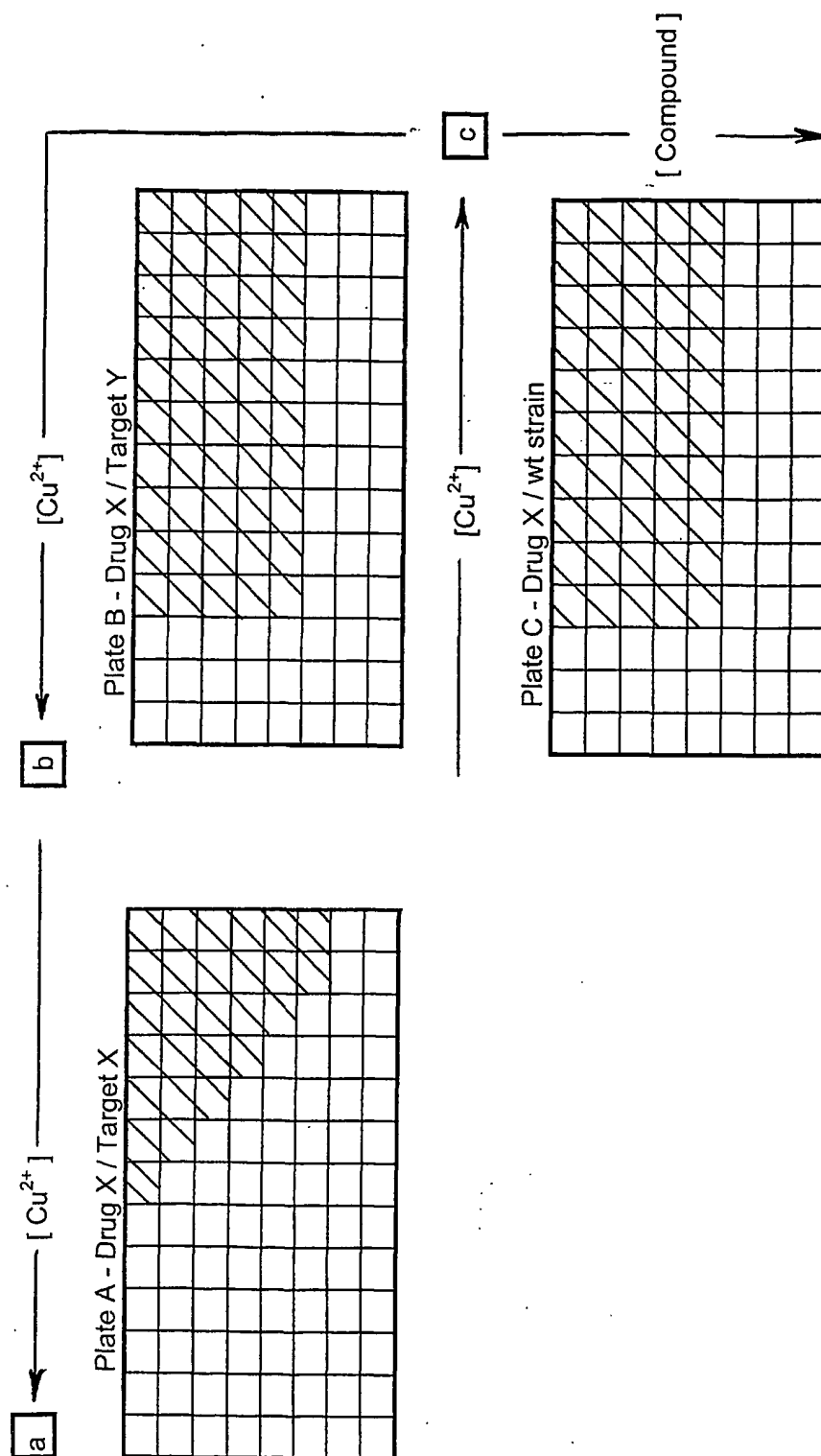
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# FIG. 12

$\mu$ g/ml Compound	mM copper sulfate												No Cu
	Cu/Drug	2	1	0.5	.25	0.125	0.063	0.032	0.016	0.008	0.004	0.0004	
0													
0.01													
0.1													
0.5													
1													
2													
8													
24													

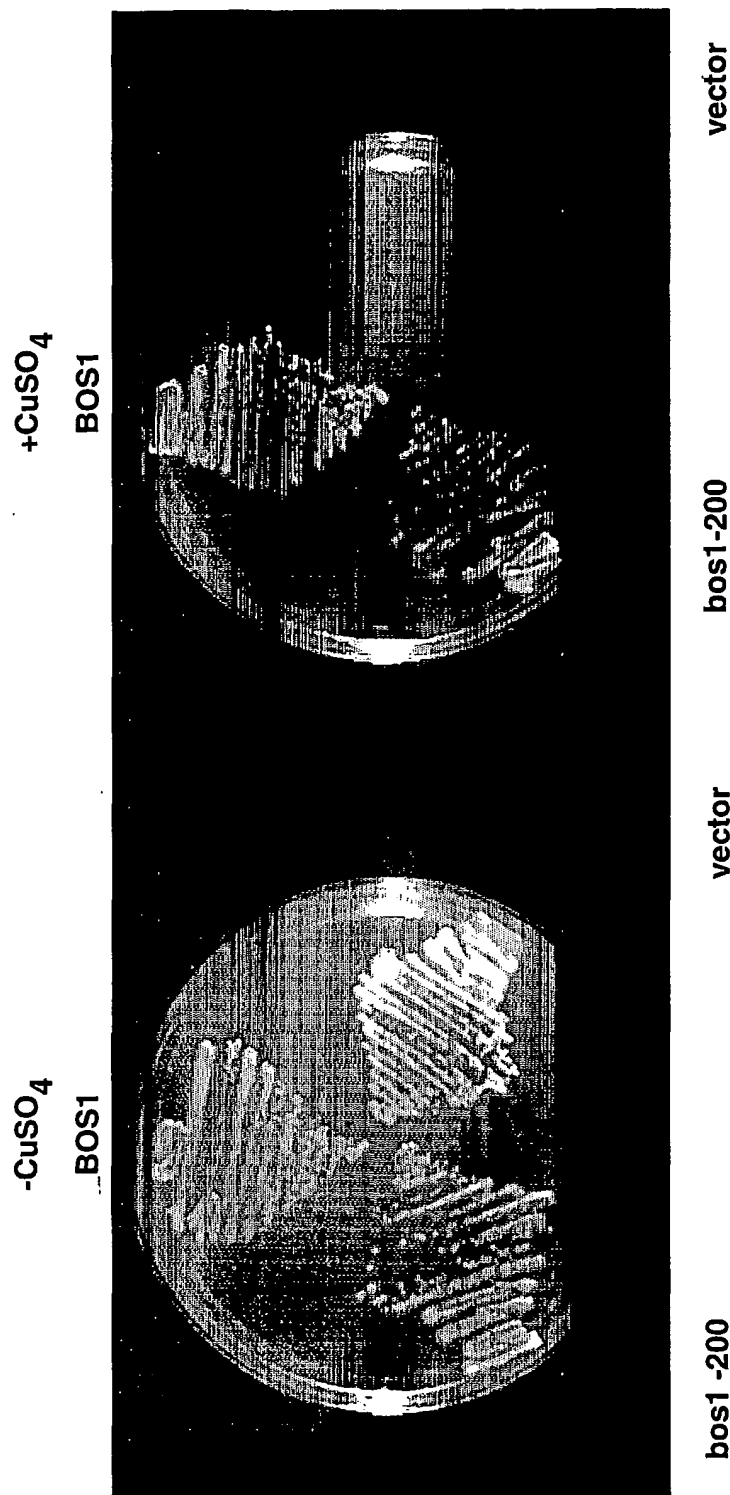
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FIG. 13



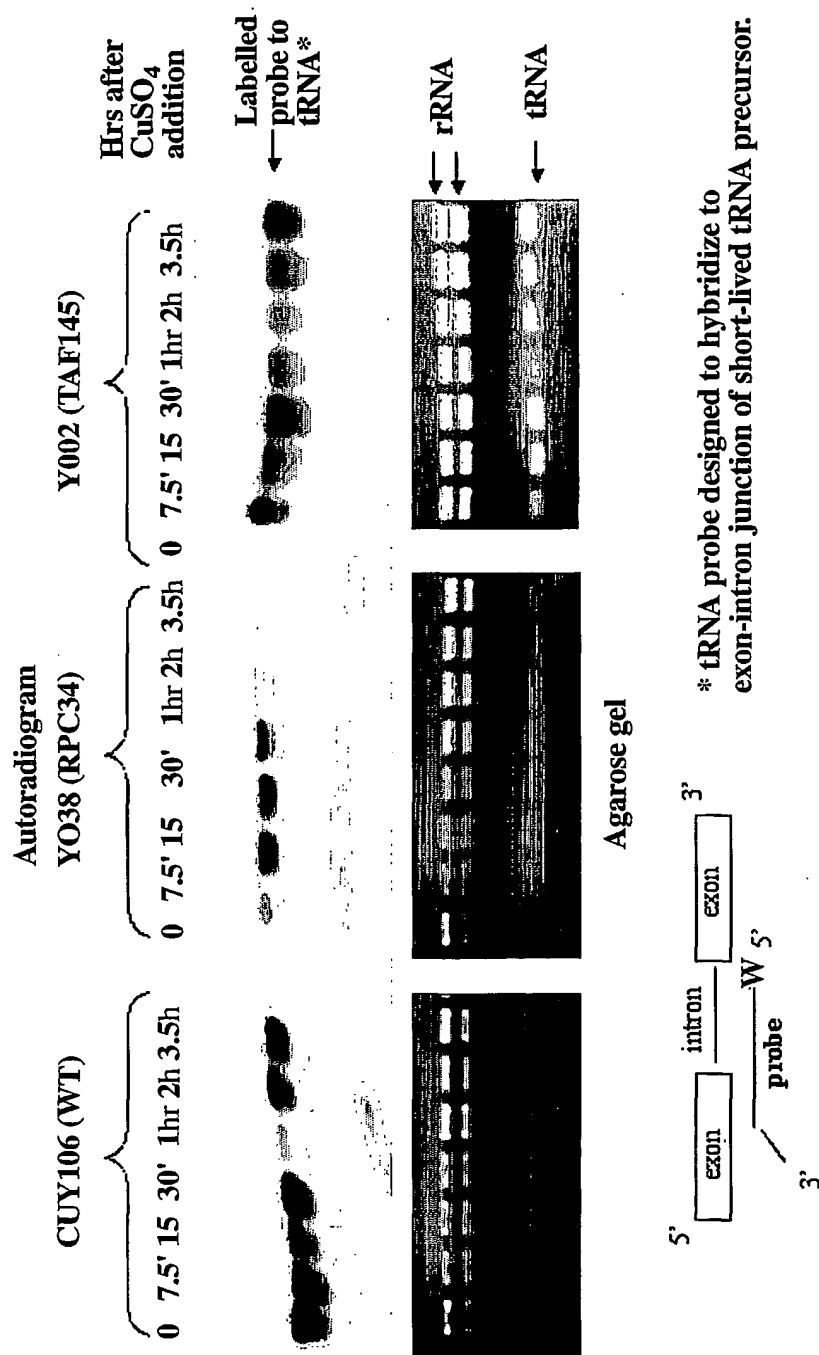
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FIG. 14



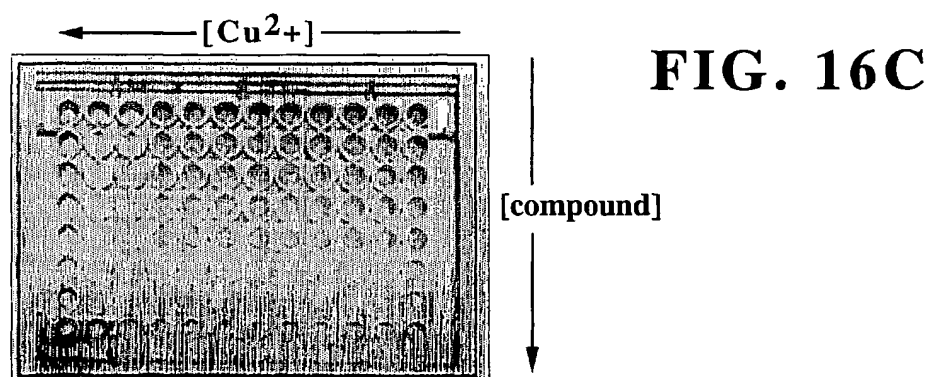
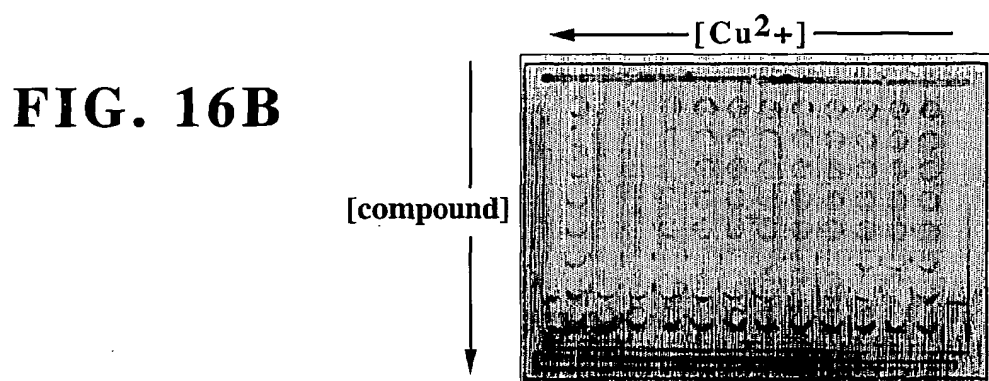
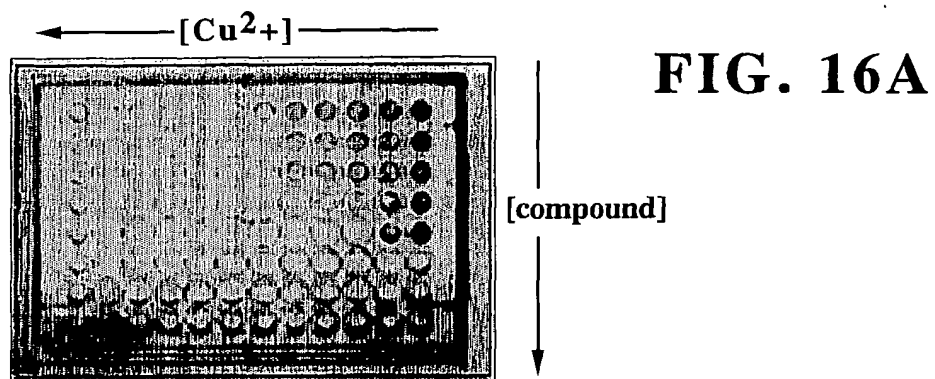
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FIG. 15





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FIG. 17A

[Conc. µg]	2	1	0.5	0.2	0.1	0.05	0.025	0.0125	0.00625	0.003125	0.0015625	0.00078125	No
0													Co
0.01													
0.1													
0.5													
1													
2													
8													
24													
Expt=	cuERG11 vs Difluconazole												

FIG. 17B

[Conc. µg]	2	1	0.5	0.2	0.1	0.05	0.025	0.0125	0.00625	0.003125	0.0015625	0.00078125	No
0													Co
0.01													
0.1													
0.5													
1													
2													
8													
24													
Expt=	CuTUB1 vs Benomyl												

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FIG. 17C

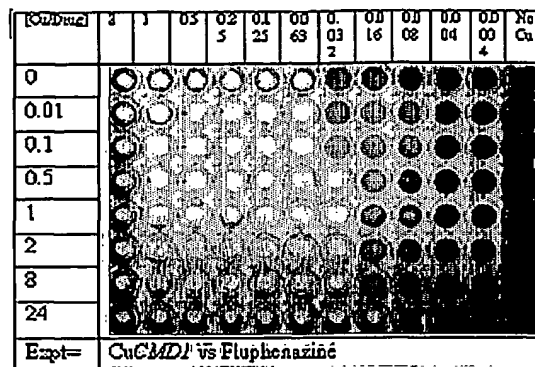
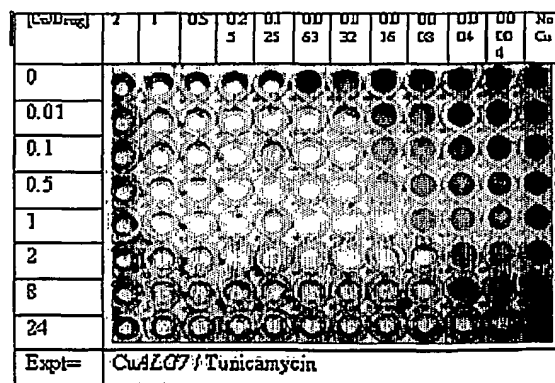


FIG. 17D



## SEQUENCE LISTING

<110> Bradley, John D.  
 Thompson, Craig M.  
 Moore, Jeffrey B.  
 Wobbe, C. Richard  
 Bailey, David A.

<120> Regulated Gene Expression in Yeast and  
 Methods of Use

<130> 0342/1D469-US4

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<141> 2000-05-18

<150> 09/404,066

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<150> 60/056,719

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19

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&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; ZM197 plasmid

&lt;400&gt; 21

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/16271

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 15/74; C12Q 1/68

US CL : 435/6, 483

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 483

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6,004,779A (BRADLEY et al.) 21 December 1999, entire document, especially columns 9-20.	1-43

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search

24 JULY 2001

Date of mailing of the international search report

29 AUG 2001

Name and mailing address of the ISA/US  
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/16271

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

US Patents, Derwent, JPO, EPO, STN (CAPLUS, Medline)

terms: yeast, cerevisiae, transcriptional, repressor, protein, polypeptide, metal ion, responsive, induc?, control?, regulat?, biomineralization, ANB1, ROX, SLF1, CYC8, LEXA, UBR1

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